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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES**

Applicant: Rajiv SHAH, et al.

Title: METHOD FOR FORMULATING A
GLUCOSE OXIDASE ENZYME
WITH A DESIRED PROPERTY OR
PROPERTIES AND GLUCOSE
OXIDASE ENZYME WITH THE
DESIRED PROPERTY

Appl. No.: 10/035,918

Filing Date: 12/28/2001

Examiner: Yong D. Pak

Art Unit: 1652

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APPEAL BRIEF UNDER 37 CFR 41.37

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Sir:

Under the provisions of 37 C.F.R. § 41.37, this Appeal Brief is being filed together with a check in the amount of \$500.00 covering the 37 C.F.R. 41.20(b)(2) appeal fee. If this fee is deemed to be insufficient, authorization is hereby given to charge any deficiency (or credit any balance) to the undersigned deposit account 50-0872.

This communication is an Appeal Brief, responsive to the Final Office Action dated July 12, 2005, concerning the above-referenced patent application.

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I. Real Party In Interest

The real party in interest for the above referenced patent application and the present Appeal is the assignee of record for the above referenced patent application, Medtronic-Minmed, Inc., as recorded at Reel 012818, Frame 0025.

II. Related Appeals And Interferences

Applicant is not aware of any related appeals, interferences or legal proceedings that would have a bearing on the Board's decision in the present Appeal.

The present patent application claims the priority filing date of U.S. Provisional Application No. 60/335,585 (now expired), for which no substantive examination on the merits was conducted by the U.S. Patent and Trademark Office.

III. Status Of The Claims

Claims 1, 3-8 and 10-54 are pending in the application.

However, claims 25-43 and 48-54 have been withdrawn from consideration by the Examiner.

Accordingly, claims 1, 3-8, 10-24 and 44-47 are pending and under consideration in the present application.

Of those claims, claims 1, 3-8, 19-24 and 44-47 are included in rejections under specific grounds identified in the Final Office Action and discussed in Sections VI. and VII., below.

The Form PTOL-326 accompanying the Final Office Action indicates that claims 1, 3-8, 10-24 and 44-47 are rejected (without specifying the ground of rejection) and a single sentence near the end of the Final Office Action states that "none of the claims are allowable" (Final Office Action dated July 12, 2005, page 10, line 13). However, claims 10-18 were not listed or addressed in any of the specific grounds of rejection included in that Final Office Action.

The present appeal relates to each of the above rejections and, thus, all of the rejected claims (i.e., claims 1, 3-5, 8, 19-24 and 44-47). If any rejection is restated to include claims 10-18, the present appeal also relates to those claims.

IV. Status Of Amendments

No amendments have been filed, subsequent to the Final Office Action of July 12, 2005.

V. Summary Of Claimed Subject Matter

Embodiments of the present invention relate, generally, to a method employing directed evolution techniques for formulating a glucose oxidase enzyme having peroxide-resistant characteristics for use, by way of example, in a sensing device.

An example implantable sensing system contains a sensing device that is inserted into a vein, an artery, or any other part of a human body where it could sense a desired parameter of the implant environment. An enzyme may be placed inside of the sensing device and employed for sensing. If the device is a glucose-sensing device, then a combination of glucose oxidase (GOx) and human serum albumin (HSA) may be utilized to form a sensor protein. During operation in a sensing device, glucose oxidase reacts with oxygen and oxidizes. The oxidation of glucose oxidase results in the formation of a hydroperoxy adduct, which transforms into hydrogen peroxide.

An obstacle to creating sensors that are long-lived and stable over time has been that glucose oxidase, when immobilized (e.g., for use in a sensor), undergoes oxidative inactivation by the aforementioned hydrogen peroxide over time. Since the lifetime of glucose sensors primarily depends on the lifetime of glucose oxidase, the effects of the peroxide on the glucose oxidase can severely limit the lifetimes of glucose sensors.

Prior processes for addressing the peroxide degradation of glucose oxidase have involved the use of additives or neutralizing agents for deactivating, removing or neutralizing peroxide. (Examples of such prior art are discussed below with respect to the Valdes et al. reference in Exhibit B.1., the Heller et al. patent in Exhibit B.3. and the Yin et al. article in Exhibit B4.) Embodiments of the present invention relate to a drastic change in direction of the state of the art

by employing directed evolution techniques to formulate a glucose oxidase gene having desired peroxide resistant properties.

Evolution under non-stress circumstances takes years. Accordingly, evolution may be manipulated in embodiments of the invention for specific enzymatic functions. In embodiments of the invention, a technique known as directed evolution is employed to evolve glucose oxidase, to formulate a glucose oxidase that possesses improved resistance to peroxide. A glucose oxidase formulated pursuant to embodiments of the present invention may improve the longevity of a biosensor in which it is employed.

According to the claims under appeal, a method comprises creating a library of mutated glucose oxidase genes. Mutations of glucose oxidase genes may be obtained by performing polymerase chain reaction techniques, error-prone polymerase chain reaction techniques or gene shuffling techniques. Each of the library of mutants is inserted into a separate expression vector. Each expression vector is inserted into a host organism where a colony can grow, thereby replicating the mutated genes.

The library of colonies is then screened for desirable peroxide resistant properties. The colonies are screened by determining whether the colonies contain active glucose oxidase and determining whether the colonies have desired peroxide resistant properties. Determining whether the colonies have desired peroxide resistant properties involves incubating the colonies in peroxide and determining whether the colonies have active glucose oxidase after incubating, including measuring a concentration of the glucose oxidase.

In one embodiment, after the screening procedure, the glucose oxidase from one or more of the screened colonies may be mutated into a second generation library of mutants. The process may then proceed again with the second generation mutations. In other embodiments, this same process may be repeated many times on subsequent generations of mutated genes until a gene is formulated with suitable properties. In one embodiment the process is repeated from two to six times. In this manner, the mutations may be refined further to provide the desired peroxide resistant properties.

Those colonies that still contain active glucose oxidase after one or more mutation and incubation procedures may possess desirable peroxide resistant qualities. Glucose oxidase from those colonies still containing active glucose oxidase may be tested for functionality, for example, by immobilizing the glucose oxidase in a sensor. In other embodiments of the invention, following at least a portion of the screening procedure, the environments of the colonies may be altered another time if desired, for example, by adding more peroxide.

The method recited in the pending claims of the present application can provide significant advantages over the prior art of record. The ability to form a stable enzyme which is peroxide resistant and which may be employed in an altered environment (oxygen free environment), such as a biosensor, can provide significant advantages in extending the life of biosensors. When used in an implanted medical device (such as an implanted blood glucose sensor), peroxide resistance and, thus, a capability for extending the life of the enzyme can provide considerable patient comfort and safety advances, for example, by reducing the frequency of surgical sensor replacements. Moreover, the ability to form enzymes with peroxide resistant properties suitable for biosensor applications in a relatively inexpensive, non-complicated and reliable process can provide significant advantages with respect to the ability to manufacture readily available supplies of the enzyme and, thus, increasing the availability of longer-life biosensors to more patients.

By a method in accordance with embodiments of the present invention, a glucose oxidase enzyme may be formulated to exhibit desired peroxide resistant properties. As such, further additives or other mechanisms for deactivating, removing or neutralizing peroxide may not be required. Thus, the disclosed method involves a distinct departure from the conventional direction of the art.

VI. Grounds Of Rejection To Be Reviewed On Appeal

Claims 1, 3-8, 19-24 and 44-47 are rejected, as follows:

1. Claims 1, 3-8, 19-24 and 44-47 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

2. Claims 1, 3-5, 8, 19-24 and 44-47 are rejected under 35 U.S.C. 103(a) as being unpatentable over Valdes et al. (Exhibit B.1) and the article titled Current Protocols in Molecular Biology (Exhibit B.2).
3. Claims 6 and 7 are rejected under 35 U.S.C. 103(a) as being unpatentable over Valdes et al. (Exhibit B.1) and the article titled Current Protocols in Molecular Biology (Exhibit B.2) and further in view of the Aldrich Catalog.
4. The Form PTOL-326 that accompanied the Final Office Action indicates that claims 1, 3-8, 10-24 and 44-47 are rejected (without specifying the ground of rejection) and a single sentence near the end of the Final Office Action states that "none of the claims are allowable" (Final Office Action dated July 12, 2005, page 10, line 13). However, claims 10-18 were not included or addressed in any of the rejections in that Final Office Action.

As noted in Section III, above, the present appeal relates to each of the above rejections and, thus, all of the rejected claims (i.e., claims 1, 3-5, 8, 19-24 and 44-47). If any rejection is restated to include claims 10-18, the present appeal also relates to those claims.

VII. Argument

1. Appeal Of Rejection Of Claims 1, 3-8, 19-24 and 44-47 Under 35 U.S.C. 112, Second Paragraph

Claims 1, 3-8, 19-24 and 44-47 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. This rejection is respectfully traversed. Applicant requests that the rejection be reversed and the rejected claims allowed in view of the following remarks.

In explaining the rejection, the Examiner stated:

"Claim 1 recites the phrase 'colonies have desired peroxide resistant properties.' Colonies having 'desired' properties conveys that the colonies having peroxide resistant properties are 'wished for' or 'longed for' and do not necessarily have 'peroxide resistant properties'. Examiner requests clarification of the above phrase."

This rejection is respectfully traversed. In particular, it is submitted that one of ordinary skill in the art would understand the cited phrase in the context of the claimed invention.

The Examiner's argument that the claims convey a wished for or longed for property and that the colonies "do not necessarily have peroxide resistant properties," is respectfully traversed. The express language of claim 1 (as quoted by the Examiner) recites that the "colonies *have* desired peroxide resistant properties" (italics added for emphasis). In that regard, the claims are specific and definite with regard to whether or not the colonies *have* the desired peroxide resistant property (and do not connote that the desired property is merely wished for or longed for, but may not be achieved).

The term "desired" is used, in the context of being a goal that is pre-defined or pre-conceived by the user. The artisan practicing the present invention would not do so in the dark, but, instead, would have a pre-defined goal (desire) in mind. Thus, the claims refer to "desired peroxide resistant properties" in a manner synonymous with "pre-defined peroxide resistant properties." It is respectfully submitted that one of ordinary skill in the art would be able to define desired peroxide resistant properties and, with the present application as a guide, determine whether the screened colonies have such desired peroxide resistant properties. In that regard, it is further respectfully submitted that the rejection of claims 1, 3-8, 19-24 and 44-47 under 35 U.S.C. 112, second paragraph, is improper and should be reversed.

2. Appeal Of Rejection Of Claims 1, 3,-5, 8, 19-24 and 44-47 Under 35 U.S.C. 103(a)

Claims 1, 3-5, 8, 19-24 and 44-47 are rejected under 35 U.S.C. 103(a) as being unpatentable over Valdes et al. and Current Protocols in Molecular Biology. This rejection is respectfully traversed. Applicant requests that the rejection be reversed and the rejected claims allowed over Valdes et al. and Current Protocols in Molecular Biology, in view of the following remarks.

Claim 1 recites a method for formulating an enzyme that is not disclosed by either Valdes et al. or in the Current Protocols in Molecular Biology article. Also, one of ordinary skill in the art, at the time of the present invention, would not have found it obvious to look to and employ

procedures for generating and screening mutations of a DNA sequence as described by the Current Protocols in Molecular Biology article to address the glucose oxidase degradation described by Valdes et al., because:

- a. None of the prior art of record provides any motivation or suggestion for the combination of the Valdes et al and the Current Protocols in Molecular Biology articles, as proposed by the Examiner;
- b. Valdes et al. and other prior art references of record teach away from a combination of the Valdes et al and the Current Protocols in Molecular Biology articles, as proposed by the Examiner;
- c. The rejection is based on an erroneous characterization of the Current Protocols in Molecular Biology article as disclosing the creation of a library of mutated glucose oxidase genes; and
- d. Each of dependent claims 3-5, 8, 19-24 and 44-47 recite further features that distinguish those claims from the prior art.

Each of those grounds for Appeal is discussed in the following subsections a-d.

a. The Rejection Is Improper Because Of Lack Of Motivation To Combine.

A method as recited in claim 1 is neither described nor suggested by either of the Valdes et al. or the Current Protocols in Molecular Biology references. For example, neither the Valdes et al. reference nor the Current Protocols in Molecular Biology reference describes or suggests formulating a glucose oxidase enzyme by “creating a library of *mutated glucose oxidase* genes” or otherwise mutating glucose oxidases. Similarly, neither of those references describe or suggest “introducing each *mutated glucose oxidase* gene of the library into separate expression vectors”, “inserting the expression vectors into host organisms”, “growing colonies of the host organisms” and “screening the colonies for desirable properties by determining whether the colonies contain active *glucose oxidase* and determining whether the colonies have desired *peroxide resistant properties*.” (See claim 1 in Appendix A, italics added for emphasis).

The Examiner has acknowledged that “Valdes et al. does not teach a method of generating a mutant glucose oxidase genes and screening for mutated glucose oxidases which are

resistant to degradation in the presence of hydrogen peroxide.” (Final Office Action dated July 12, 2005, pg. 4, ll. 16-18.) However, the Examiner argues that the Current Protocols in Molecular Biology reference “teaches many different protocols in generating a library of mutated *glucose oxidase* genes via error-prone PCR and gene shuffling, screening, selecting and isolating mutated genes and expression of the mutant protein (Chapter 3, 5-6, 8 and 10).” (italics added for emphasis)(Final Office Action, pg. 4, l. 22 to pg. 5, l. 3.)¹

More specifically, the Examiner argues that “it would have been obvious to one of ordinary skill to incubate colonies comprising said genes with hydrogen peroxide and determine if the encoded protein retain enzymatic activity, indicating their resistance to hydrogen peroxide.” (Final Office Action, pg. 5, ll. 5-9.) However, the Examiner cites no suggestion or motivation in either the Valdes et al. reference or the Current Protocols in Molecular Biology reference (or any other prior art) for incubating mutated colonies of glucose oxidase with hydrogen peroxide. Instead, the Examiner makes the following conclusory statement that is not supported by the cited prior art, but is derived from the teaching of the present application: “[o]ne of ordinary skill in the art would have been motivated to mutagenize the protein [of Valdes et al.] in order to screen for mutants which are able to retain enzymatic activity in glucose sensors in the presence of hydrogen peroxide.” (Final Office Action, pg. 5, ll. 5-12.) The only teaching or suggestion of record of mutating *glucose oxidase* and screening for *hydrogen peroxide resistant properties* is that of the present disclosure.

In fact, neither Valdes et al. nor the Current Protocols in Molecular Biology references provide any motivation or suggestion for creating a library of mutated glucose oxidase genes and screening colonies for active glucose oxidase and desired peroxide resistant properties. Indeed, as discussed in Section VII.2.b., below, Valdes et al. teach away from such methods by, instead, referring to conventional procedures (using additives for deactivating or destroying hydrogen peroxide and, thus, teach away from such a method, as follows:

“A long term remedy of the degradation of GOD by H₂O₂ could be the immobilization and attachment of the enzyme to a support that deactivates H₂O₂, as it is being produced.

¹ As discussed in Section VII.1.c., below, the Current Protocols in Molecular Biology reference does not teach of mutating glucose oxidase.

Such as study was conducted by Cho², using the peroxide decomposition catalyst, activated carbon. In a study conducted by Carter¹⁹, the best results were obtained with activated carbon, impregnated with ruthenium. This combination was able to destroy hydrogen peroxide and stabilized the enzyme.” (Valdes et al., pg. 375, col. 1, l.18 to col. 2, l. 6.)

While the Valdes et al. and Current Protocols in Molecular Biology references, themselves, provide no motivation or suggestion, the Examiner argues that a “reasonable expectation of success” provides motivation, as follows:

“One of ordinary skill in the art would have had a reasonable expectation of success in making the mutant glucose oxidases resistant to peroxide degradation since Current Protocols in Molecular Biology demonstrates the success of random mutagenesis employing different PCR techniques and teaches different methods of screening, selecting and isolating mutated gene and its encoded protein and since Valdes et al. teaches that the degradation of glucose oxidase in glucose sensors is due to hydrogen peroxide.” (Final Office Action, pg. 5, ll. 15-21.)

However, without the present disclosure as a guide, one of ordinary skill in the art would not have selected a DNA mutation and screening process described by the Current Protocols in Molecular Biology reference to mutate glucose oxidase genes, much less to screen colonies for peroxide resistant properties. The Examiner’s conclusory statements of motivation to combine and the Examiner’s argument of “reasonable expectation of success” fail to address the significant issue of why one skilled in the art would have been motivated to select a DNA mutation and screening process described by the Current Protocols in Molecular Biology, to change the direction taken by those most skilled in the prior art as described by Valdes et al.

When patentability turns on the question of obviousness, the search for and analysis of the prior art includes evidence relevant to the finding of whether there is a teaching, motivation, or suggestion to select and combine the references relied on as evidence of obviousness. (underline added for emphasis.) See, e.g., *McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1351—52, 60 USPQ2d 1001, 1008 (Fed. Cir. 2001) (“the central question is whether there is reason to combine [the] references,” a question of fact drawing on the *Graham* factors).

Conclusory statements that prior art references provide motivation to combine, or statements of motivation derived from the Applicant’s own specification, are not sufficient to set forth a *prima facie* case of obviousness. “The factual inquiry whether to combine references

must be thorough and searching.” *Id.* It must be based on objective evidence of record. This precedent has been reinforced in myriad decisions. *See, e.g., Brown & Williamson Tobacco Corp. v. Philip Morris Inc.*, 229 F.3d 1120, 1124—25, 56 USPQ2d 1456, 1459 (Fed. Cir. 2000) (“a showing of a suggestion, teaching, or motivation to combine the prior art references is an ‘essential component of an obviousness holding’”) (quoting *C.R. Bard, Inc., v. M3 Systems, Inc.*, 157 F.3d 1340, 1352, 48 USPQ2d 1225, 1232 (Fed. Cir. 1998)); *In re Dembiczak*, 175 F.3d 994, 999, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999) (“Our case law makes clear that the best defense against the subtle but powerful attraction of a hindsight-based obviousness analysis is rigorous application of the requirement for a showing of the teaching or motivation to combine prior art references.”); *In re Dance*, 160 F.3d 1339, 1343, 48 USPQ2d 1635, 1637 (Fed. Cir. 1998) (there must be some motivation, suggestion, or teaching of the desirability of making the specific combination that was made by the applicant); *In re Fine*, 837 F.2d 1071, 1075, 5 USPQ2d 1596, 1600 (Fed. Cir. 1988) (“‘teachings of references can be combined *only* if there is some suggestion or incentive to do so.’”) (emphasis in original) (quoting *ACS Hosp. Sys., Inc. v. Montefiore Hosp.*, 732 F.2d 1572, 1577, 221 USPQ 929, 933 (Fed. Cir. 1984)).

As noted above, the Examiner has not shown any motivation or suggestion in the prior art that would have led one skilled in the art to select a DNA mutation and screening process described by the Current Protocols in Molecular Biology for creating a library of mutated glucose oxidase and screening colonies for peroxide resistant properties. In fact, as described in Section III.1.b., below, the primary reference relied upon by the Examiner (Valdes et al) and other prior art of record show that a selection of a mutation and screening process would have been a drastic diversion from the direction taken by those most skilled in the prior art.

The legal authority expresses the requirement for a showing of specificity in the prior art of motivation to select components to combine. *See, e.g., In re Kotzab*, 217 F.3d 1365, 1371, 55 USPQ2d 1313, 1317 (Fed. Cir. 2000) (“particular findings must be made as to the reason the skilled artisan, with no knowledge of the claimed invention, would have selected these components for combination in the manner claimed”); *In re Rouffet*, 149 F.3d 1350, 1359, 47 USPQ2d 1453, 1459 (Fed. Cir. 1998) (“even when the level of skill in the art is high, the Board must identify specifically the principle, known to one of ordinary skill, that suggests the claimed

combination. In other words, the Board must explain the reasons one of ordinary skill in the art would have been motivated to select the references and to combine them to render the claimed invention obvious.”); *In re Fritch*, 972 F.2d 1260, 1265, 23 USPQ2d 1780, 1783 (Fed. Cir. 1992) (the examiner can satisfy the burden of showing obviousness of the combination “only by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings of the references”).

In response to Applicant’s remarks regarding the nonobviousness to combine the Current Protocols in Molecular Biology with the Valdes et al. reference, the Examiner argued that Valdes et al. teaches that “the enzyme used to detect glucose, glucose oxidase, degrades over time due to peroxide degradation” and that “[o]ne of ordinary skill in the art ... would have been motivated to reduce degradation of glucose oxidase, either by removing or neutralizing peroxide *or using mutants which are resistant to peroxide.*” (Italics added for emphasis.)(Final Office Action, pg. 6, ll. 13-17). The Examiner’s reference to removing or neutralizing peroxide appears to be taken from the teachings of Valdes et al., Heller or Yin references (Exhibits B.1., B.4 and B.5 herein, each of which are described below), which represent conventional manners of addressing peroxide degradation of glucose oxidase. However, the Examiner’s reference to “using mutants which are resistant to peroxide” is based on hindsight derived from the present application. The Current Protocols in Molecular Biology article makes no reference to glucose oxidase and, as discussed above, Valdes et al. refers to conventional (additive) processes for neutralizing peroxide. The only teaching of record of *mutating glucose oxidase and screening colonies for peroxide resistant properties* is that of the present application. The Examiner’s argument that one skilled in the art would have been motivated to “use mutants which are resistant to peroxide,” does not identify where any such motivation would have been derived (without using the present application as a guide).

The Examiner further argues that “[w]hile at one time one of ordinary skill in the art would have addressed the peroxide degradation by such methods [of removing peroxide], the art of biotechnology is constantly changing” and “[m]ethods in generating random mutagenesis via PCR and screening for mutant having desired properties are very well known and have been

widely practiced, as taught by Current Protocols in Molecular Biology.” (Final Office Action, pg. 7, ll. 1-5.) However, the Current Protocols in Molecular Biology article was published well before (about 3 years before) the Valdes et al. reference. The fact that the later-published Valdes et al. reference makes no mention of using random mutagenesis and screening (but, instead, refers to conventional manners of addressing peroxide degradation of glucose oxidase) is evidence that it would not have been obvious to select a process as described by the Current Protocols in Molecular Biology reference to address peroxide degradation of glucose oxidase. Had such been obvious, then Valdes et al. would likely have referred to mutation and screening processes, instead of merely referring to conventional processes involving additives or a neutralizing support base.

Because the Examiner has not shown any motivation or suggestion in the prior art that would have led one skilled in the art to select a DNA mutation and screening process described by the Current Protocols in Molecular Biology to combine with the Valdes et al. reference, the Examiner has not raised a *prima facie* case of obviousness. Therefore, the rejection of 1, 3-5, 8, 19-24 and 44-47 under 35 U.S.C. 103(a) is respectfully traversed.

b. The Prior Art Teaches Away From The Combination.

The Examiner’s argument that a “reasonable expectation of success” would have motivated the combination (Final Office Action, pg. 5, ll. 15-21), is contrary to the express teachings of the prior art. The prior art teaches that those most skilled in the art were taking a wholly different direction to address peroxide degradation of glucose oxidase and, thus, would have found it unreasonable (not reasonable) to change the course of direction from that of the state of the art.

In particular, Valdes et al. refers to conventional, known “additive” methods for addressing peroxide degradation of glucose oxidase. Mutation of glucose oxidase genes and screening of mutated glucose oxidase for hydrogen peroxide resistant properties would have been a drastic departure from the state of the art and, without the benefit of the present specification as a guide, would not have been obvious to one of ordinary skill in the art.

More specifically, Valdes et al. refer to completely different directions taken by those most skilled in the art, whereby the glucose oxidase enzyme is immobilized and attached to a support that deactivates peroxide. "A reference may be said to teach away when a person of ordinary skill, upon reading the reference, ... would be led in a direction divergent from the path that was taken by the applicant." *Tec Air, Inc. v. Denso Mfg. Mich. Inc.*, 192 F.3d 1353, 1360, 52 USPQ2d 1294, 1298 (Fed. Cir. 1999). Valdes et al., directly refers the reader to conventional methods of addressing peroxide degradation of glucose oxidase that employ additives for destroying or neutralizing peroxide (which is quite different from creating a library of mutated genes and screening for desired peroxide resistant properties). As taught by Valdes, et al:

"A long term remedy of the degradation of GOD by H_2O_2 could be the immobilization and attachment of the enzyme to a support that deactivates H_2O_2 , as it is being produced. Such as study was conducted by Cho², using the peroxide decomposition catalyst, activated carbon. In a study conducted by Carter¹⁹, the best results were obtained with activated carbon, impregnated with ruthenium. This combination was able to destroy hydrogen peroxide and stabilized the enzyme." (Valdes et al., pg. 375, col. 1, l.18 to col. 2, l. 6.)

Not only does Valdes et al. fail to teach or suggest to mutate glucose oxidase and screen mutated glucose oxidase for peroxide resistance properties, but, in the above-quoted statement, Valdes et al. further teaches to use other, very different procedures (conventional in the art) to address degradation effects of peroxide on glucose oxidase. Thus, the Valdes et al. reference shows that the direction taken by those most skilled in the art involved employing materials, additives, or the like that deactivate peroxide.

Additional prior art of record also describes conventional "additive" processes for removing or neutralizing peroxide such as by adding an antioxidant or peroxidase to the glucose oxidase to break down peroxide or by coating the glucose oxidase enzyme with a protective coating, including U.S. Patent No. 6,689,265 to Heller et al. (Exhibit B.3) and the article titled "Glucose ENFET doped with MnO_2 powder" by Yin et al (Exhibit B.4.). Those prior art references further emphasize that the direction taken by those skilled in the art for addressing the peroxide degradation of glucose oxidase is wholly different from the direction of the present invention. In U.S. Patent No. 6,689,265 to Heller et al., a peroxide generating enzyme may include a sufficiently thick, natural, electrically insulating protein or glycoprotein layer. (See

column 6, lines 59-67 of the Heller et al. patent, Exhibit B.3.) Heller et al. also disclose an alternative embodiment in which a peroxide generating enzyme is immobilized in a non-conducting inorganic or organic polymeric matrix. (See column 7, lines 3-11 of the Heller et al. patent, Exhibit B.3.) Also, Heller et al. describe a first layer enzyme 11 (peroxidase) that reduces peroxide generated from a second layer (glucose oxidase layer) 13. The Yin et al. article describes the addition of MnO_2 to catalyze peroxide and produce water and oxygen therefrom. (Yin, Exhibit B.4, Abstract and pg. 188, col. 1, ll. 20-34.)

Thus, both the Heller et al. patent and the Yin et al. article show that the direction taken by those skilled in the art is to provide additives or complex multi-layer sensor structures to remove hydrogen peroxide. These references, in addition to Valdes et al.'s express references to conventional uses of additives, show that those skilled in the art were not considering mutating glucose oxidase genes and growing and screening colonies for peroxide resistance, but instead were attempting to address the peroxide production issue by removing or neutralizing peroxide with additives (not by altering the glucose oxidase). The state and direction of the prior art, as evidenced by Valdes et al., Heller et al. and Yin et al., was a wholly different direction than that taken by the present Applicants (including creating a library of mutated glucose oxidase enzyme genes and screening colonies for desirable properties by determining whether the colonies contain active glucose oxidase and determining whether the colonies have desired peroxide resistant properties.)

The fact that the primary reference (Valdes et al.) teach away from the claimed invention and the combination suggested by the Examiner, shows that a *prima facie* case of obviousness has not been raised. Numerous Federal Circuit decisions recognize that an invention will not be deemed obvious in a patent law sense when one or more prior art references "teach away" from the invention. For example, the Federal Circuit stated "as a useful general rule, that references that teach away cannot serve to create a *prima facie* case of obviousness." *McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1354, 60 USPQ2d 1001 (Fed. Cir. 2001).

Furthermore, "an applicant may rebut a *prima facie* case of obviousness by showing that the prior art teaches away from the claimed invention in any material respect." *In re Peterson*, 315 F.3d 1325, 1331, 65 USPQ2d 1379 (Fed. Cir. 2003). Also see, *Gillette Co. v. S.C. Johnson*

& Sons, Inc., 919 F.2d 720, 724, 16 USPQ2d 1923, 1927 (Fed. Cir. 1990)(the closest prior art reference “would likely discourage the art worker from attempting the substitution suggested by [the inventor/patentee]”) and *Singh v. Brake*, 317 F.3d 1334, 1346, 65 USPQ2d 1641 (Fed. Cir. 2003)(“whether or not a reference ‘teaches away’ from a claimed invention” is “relevant in determining whether or not a claimed invention would have been obvious”).

Without the present disclosure as a guide, one of ordinary skill in the art would not have found Valdes et al.’s discussion of the degradation of glucose oxidase as a prompt or suggestion to employ a mutation process as described in the Current Protocols in Molecular Biology reference to mutate glucose oxidase genes. Instead, as noted above, one of ordinary skill in the art would have looked to conventional manners of removing peroxide, such as additives for removing or neutralizing peroxide. Accordingly, the rejection of 1, 3-5, 8, 19-24 and 44-47 under 35 U.S.C. 103(a) is further respectfully traversed.

c. The rejection is based on an erroneous characterization of the Current Protocols in Molecular Biology reference.

The rejection is based on an erroneous characterization of the Current Protocols in Molecular Biology reference as disclosing the creation of a library of *mutated glucose oxidase genes*. As the Valdes et al. reference also fails to disclose such a library, the combination of the Valdes et al. and the Current Protocols in Molecular Biology references proposed by the Examiner (which combination is respectfully traversed for reasons discussed below) would not have resulted in the presently claimed invention.

As discussed above, the Examiner has acknowledged that “Valdes et al. does not teach a method of generating a mutant glucose oxidase genes and screening for mutated glucose oxidases which are resistant to degradation in the presence of hydrogen peroxide.” (Final Office Action dated July 12, 2005, pg. 4, ll. 16-18.) However, the Examiner argues that the Current Protocols in Molecular Biology reference “teaches many different protocols in generating a library of mutated glucose oxidase genes via error-prone PCR and gene shuffling, screening, selecting and isolating mutated genes and expression of the mutant protein (Chapter 3, 5-6, 8 and 10).” (Final Office Action, pg. 4, l. 22 to pg. 5, l. 3.)

To the contrary, in reviewing the lengthy, cited chapters of the Current Protocols in Molecular Biology reference, Applicant found no reference to generating a library of mutated glucose oxidase genes, much less screening such mutated genes for peroxide resistance properties. Thus, it appears that the rejection is partially based on a mis-characterization of the Current Protocols in Molecular Biology reference.

Thus, neither the Valdes et al. reference nor the Current Protocols in Molecular Biology reference describe or suggest “creating a library of mutated glucose oxidase genes;” “introducing each mutated glucose oxidase gene of the library into separate expression vectors”, “inserting the expression vectors into host organisms”, “growing colonies of the host organisms” and “screening the colonies for desirable properties by determining whether the colonies contain active glucose oxidase and determining whether the colonies have desired peroxide resistant properties.” The Examiner’s proposal to combine the teachings of those references (which Applicant traverses above), therefore, would not lead to a method involving those features. Accordingly, the rejection of 1, 3-5, 8, 19-24 and 44-47 under 35 U.S.C. 103(a) is further respectfully traversed.

d. Each of dependent claims 3-5, 8, 19-24 and 44-47 recite further features that distinguish those claims from the prior art.

Each of dependent claims 3-5, 8, 19-24 and 44-47 recite further features that distinguish those claims from the prior art. In particular, each of those claims recites features relating to colonies of mutated glucose oxidase gene. As described above, neither Valdes et al. nor the Current Protocols in Molecular Biology references describe or suggest mutating glucose oxidase. In that regard, those references also do not disclose or suggest the additional processing recited in dependent claims 3-5, 8, 20-24 and 44-47, including:

1. “screening the colonies for desirable properties further comprises testing glucose oxidase from the colonies for functionality” (claim 3);
2. “determining whether the colonies have peroxide-resistant properties is only performed if results of determining whether the colonies contain active glucose oxidase are positive” (claim 4);

3. “testing glucose oxidase from the colonies for functionality is only performed if results of determining whether the colonies contain active glucose oxidase are positive and if results of determining whether the colonies have peroxide resistant properties are positive” (claim 5);
4. “creating at least one mutated glucose oxidase gene comprises employing polymerase chain reaction techniques to create at least one mutated glucose oxidase gene” (claim 20);
5. “employing error-prone polymerase chain reaction techniques to create at least one mutated glucose oxidase gene” (claim 21);
6. “employing gene shuffling techniques to create at least one mutated glucose oxidase gene” (claim 22);
7. “creating a next generation of mutated glucose oxidase genes after screening the colonies for desirable properties” (claim 23); and
8. “creating a next generation of mutated glucose oxidase genes is repeated approximately 2 to 6 times” (claim 24).

Because neither Valdes et al. nor the Current Protocols in Molecular Biology references describe or suggest mutating glucose oxidase, those references also do not disclose or suggest the additional processing recited in dependent claims 3-5, 8, 20-24 and 44-47. The rejection of claims 3-5, 8, 20-24 and 44-47 is, therefore respectfully traversed and should be reversed.

3. Appeal Of Rejection Of Claims 6 and 7 Under 35 U.S.C. 103(a)

Claims 6 and 7 are rejected under 35 U.S.C. 103(a) as being unpatentable over Valdes et al. and Current Protocols in Molecular Biology and further in view of Byalina et al. This rejection is respectfully traversed, at least for reasons as discussed above with regard to independent claim 1.

In particular, claims 6 and 7 are dependent (directly and indirectly) on claim 1. Due to their dependencies, the distinctions over the prior art of record discussed above with respect to claim 1 apply to claims 6 and 7, as well. Moreover, it is noted that the Byalina et al. reference does not address the distinctions over the references discussed above with respect to claim 1. Byalina et al neither teach nor suggest mutating glucose oxidase genes or of screening colonies of glucose oxidase for peroxide resistance.

The Bylina et al. reference was cited by the Examiner for a discussion of screening assays of colonies containing mutant proteins. However, Bylina et al. does not teach or suggest mutating glucose oxidase genes, screening colonies for peroxide resistance or other aspects of the claimed method for formulating an enzyme. Thus, Bylina does not address the distinctions noted above with respect to claim 1.

Each of dependent claims 6 and 7 recite further features that distinguish those claims from the prior art. In particular, each of those claims recites features relating to colonies of mutated glucose oxidase gene. As described above, neither Valdes et al. nor the Current Protocols in Molecular Biology references describe or suggest mutating glucose oxidase. In that regard, those references also do not disclose or suggest the additional processing recited in dependent claims 6 and 7, including “determining whether the colonies have active glucose oxidase comprises employing a substance that changes color in the presence of active glucose oxidase” (claim 6) and using leuco-crystal-violet as the substance (claim 7).

Because neither Valdes et al., the Current Protocols in Molecular Biology nor the Bylina et al. references describe or suggest mutating glucose oxidase, those references also do not disclose or suggest the additional processing recited in dependent claims 6 and 7. The rejection of claims 6 and 7 is, therefore respectfully traversed and should be reversed.

4. Claims 10-18

Claims 10-18 were not expressly included or addressed in any of the rejections in the Final Office Action. Thus, it appears that claims 10-18 are not under rejection.

However, the cover Form PTOL-326 that accompanied the Final Office Action indicates that claims 1, 3-8, 10-24 and 44-47 are rejected (without specifying the ground of rejection) and a single sentence near the end of the Final Office Action states that “none of the claims are allowable” (Final Office Action dated July 12, 2005, page 10, line 13). If any rejection is restated to include claims 10-18, the present appeal also relates to those claims.

Claims 10-18 are each dependent, indirectly, on claim 1 and, thus, include each of the features of claim 1. Because claim 1 is patentable over the prior art of record for reasons

discussed above, it is also submitted that claims 10-18 are also patentable at least for reasons as discussed above with respect to claim 1.

In addition, each of claims 10-18 recite further features that distinguish those claims from the prior art. In particular, each of those claims recites features relating to colonies of mutated glucose oxidase gene. As described above, neither Valdes et al. nor the Current Protocols in Molecular Biology references describe or suggest mutating glucose oxidase. In that regard, those references also do not disclose or suggest the additional processing recited in dependent claims 10-18, including:

1. "testing glucose oxidase from the colonies for functionality comprises employing glucose oxidase from the colonies in sensors" (claim 10);
2. "extracting glucose oxidase from the colonies; immobilizing the glucose oxidase after extracting the glucose oxidase from the colonies; placing the immobilized glucose oxidase in a sensor; and testing the sensor" (claim 11);
3. "employing an ionic column to extract glucose oxidase from the colonies" (claim 12);
4. "removing the glucose oxidase from the colonies; purifying the glucose oxidase; and characterizing the glucose oxidase" (claim 13);
5. "grinding the colonies in a homogenizer into cell components" (claim 14);
6. "fractionating the cell components employing centrifugation and differential solubility after grinding the colonies in a homogenizer" (claim 15);
7. "disrupting the colonies into cell components via sonication" (claim 16);
8. "fractionating the cell components employing centrifugation and differential solubility after disrupting the colonies via sonication" (claim 17);
9. "purifying the glucose oxidase by employing chromatography methods" (claim 18).

Because neither Valdes et al. nor the Current Protocols in Molecular Biology references describe or suggest mutating glucose oxidase, those references also do not disclose or suggest the additional processing recited in dependent claims 10-18. The rejection of claims 10-18 is, therefore respectfully traversed and should be reversed.

VIII. Conclusion

In view of the foregoing, it is respectfully submitted that claims 1, 3-8, 10-24 and 44-47 are in condition for allowance and the application should be allowed in its present form. In particular, it is respectfully submitted that the presently pending rejections of claims 1, 3-8, 19-24 and 44-47 are improper and should be reversed for reasons as discussed above. In that regard, each of claims 1, 3-8, 19-24 and 44-47 is in condition for allowance. While no formal rejection of claims 10-18 was included in the Final Office Action, if any rejection is restated to include claims 10-18, then that rejection should be reversed for reasons as discussed above, placing claims 10-18 in condition for allowance.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 50-0872. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 50-0872. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 50-0872.

Respectfully submitted,

Date: December 7, 2005

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By: 

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APPENDIX A

Claims Appendix

1. (Currently Amended) A method for formulating an enzyme comprising:
obtaining a library of glucose oxidase genes;
creating a library of mutated glucose oxidase genes;
introducing each mutated glucose oxidase gene of the library into separate expression
vectors;
inserting the expression vectors into host organisms;
growing colonies of the host organisms; and
screening the colonies for desirable properties by determining whether the colonies
contain active glucose oxidase and determining whether the colonies have desired peroxide
resistant properties,
wherein determining whether the colonies have peroxide resistant properties comprises:
incubating the colonies in peroxide; and
determining whether the colonies have active glucose oxidase after incubating the
colonies in peroxide, and
wherein determining whether the colonies contain active glucose oxidase comprises:
measuring a concentration of the glucose oxidase.
2. Cancelled.
3. (Previously presented) A method for formulating an enzyme according to claim 1,
wherein screening the colonies for desirable properties further comprises testing glucose oxidase
from the colonies for functionality.
4. (Original) A method for formulating an enzyme according to claim 1, wherein
determining whether the colonies have peroxide resistant properties is only performed if results
of determining whether the colonies contain active glucose oxidase are positive.
5. (Original) A method for formulating an enzyme according to claim 3, wherein
testing glucose oxidase from the colonies for functionality is only performed if results of

determining whether the colonies contain active glucose oxidase are positive and if results of determining whether the colonies have peroxide resistant properties are positive.

6. (Previously presented) A method for formulating an enzyme according to claim 1, wherein determining whether the colonies have active glucose oxidase comprises employing a substance that changes color in the presence of active glucose oxidase.

7. (Original) A method for formulating an enzyme according to claim 6, wherein the substance is leuco-crystal-violet.

8. (Previously presented) A method for formulating an enzyme according to claim 1, wherein determining whether the colonies have active glucose oxidase comprises checking for fluorescence.

9. Cancelled.

10. (Previously presented) A method for formulating an enzyme according to claim 2 3, wherein testing glucose oxidase from the colonies for functionality comprises employing glucose oxidase from the colonies in sensors.

11. (Original) A method for formulating an enzyme according to claim 10, wherein employing glucose oxidase from the colonies in sensors comprises:
extracting glucose oxidase from the colonies;
immobilizing the glucose oxidase after extracting the glucose oxidase from the colonies;
placing the immobilized glucose oxidase in a sensor; and
testing the sensor.

12. (Original) A method for formulating an enzyme according to claim 11, wherein extracting glucose oxidase from the colonies comprises employing an ionic column to extract glucose oxidase from the colonies.

13. (Original) A method for formulating an enzyme according to claim 11, wherein extracting glucose oxidase from the colonies comprises:

removing the glucose oxidase from the colonies;
purifying the glucose oxidase; and
characterizing the glucose oxidase.

14. (Original) A method for formulating an enzyme according to claim 13, wherein removing the glucose oxidase from the colonies comprises grinding the colonies in a homogenizer into cell components.

15. (Original) A method for formulating an enzyme according to claim 14, wherein removing the glucose oxidase from the colonies further comprises fractionating the cell components employing centrifugation and differential solubility after grinding the colonies in a homogenizer.

16. (Original) A method for formulating an enzyme according to claim 13, wherein removing the glucose oxidase from the colonies comprises disrupting the colonies into cell components via sonication.

17. (Original) A method for formulating an enzyme according to claim 16, wherein removing the glucose oxidase from the colonies further comprises fractionating the cell components employing centrifugation and differential solubility after disrupting the colonies via sonication.

18. (Original) A method for formulating an enzyme according to claim 13, wherein purifying the glucose oxidase comprises purifying the glucose oxidase by employing chromatography methods.

19. (Original) A method for formulating an enzyme according to claim 1, wherein the glucose oxidase is obtained from an organism and wherein the organism is selected from a group consisting of *Aspergillus Niger*, *Penicillium funiculosum*, *Saccharomyces cerevisiae*, and *Escherichia Coli*.

20. (Original) A method for formulating an enzyme according to claim 1, wherein creating at least one mutated glucose oxidase gene comprises employing polymerase chain reaction techniques to create at least one mutated glucose oxidase gene.

21. (Original) A method for formulating an enzyme according to claim 1, wherein creating at least one mutated glucose oxidase gene comprises employing error-prone polymerase chain reaction techniques to create at least one mutated glucose oxidase gene.

22. (Original) A method for formulating an enzyme according to claim 1, wherein creating at least one mutated glucose oxidase gene comprises employing gene shuffling techniques to create at least one mutated glucose oxidase gene.

23. (Original) A method for formulating an enzyme according to claim 1, wherein the method further comprises creating a next generation of mutated glucose oxidase genes after screening the colonies for desirable properties.

24. (Original) A method for formulating an enzyme according to claim 23, wherein creating a next generation of mutated glucose oxidase genes is repeated approximately 2 to 6 times.

25. (Withdrawn) An enzyme formulated according to the method of claim 1.

26. (Withdrawn) A method for formulating an enzyme comprising:

obtaining an organism with a glucose oxidase gene;

growing multiple colonies of the organism;

altering the environment of the colonies; and

screening the colonies to identify colonies with active glucose oxidase after altering the environment of the colonies.

27. (Withdrawn) A method for formulating an enzyme according to claim 26, wherein the organism is selected from a group consisting of *Aspergillus Niger*, *Penicillium funiculosum*, *Saccharomyces cerevisiae*, and *Escherichia Coli*.

28. (Withdrawn) A method for formulating an enzyme according to claim 26, wherein altering the environment of the colonies comprises introducing peroxide to the colonies.

29. (Withdrawn) A method for formulating an enzyme according to claim 26, wherein screening the colonies to identify colonies with active glucose oxidase comprises employing a substance that changes color in the presence of active glucose oxidase.

30. (Withdrawn) A method for formulating an enzyme according to claim 29, wherein the substance is leuco-crystal-violet.

31. (Withdrawn) A method for formulating an enzyme according to claim 30, wherein screening the colonies to identify colonies with active glucose oxidase comprises checking for fluorescence.

32. (Withdrawn) A method for formulating an enzyme according to claim 26, wherein the method further comprises testing the colonies with active glucose oxidase for functionality after screening the colonies to identify colonies with active glucose oxidase.

33. (Withdrawn) A method for formulating an enzyme according to claim 32, wherein the method further comprises continuing to alter the environments of the colonies until the colonies with active glucose oxidase are of a suitable number to proceed with testing the colonies with active glucose oxidase for functionality.

34. (Withdrawn) A method for formulating an enzyme according to claim 32, wherein testing the colonies with active glucose oxidase for functionality comprises employing glucose oxidase from the colonies in sensors.

35. (Withdrawn) A method for formulating an enzyme according to claim 32, wherein testing the colonies with active glucose oxidase for functionality comprises:

extracting glucose oxidase from the colonies;

immobilizing the glucose oxidase after extracting the glucose oxidase from the colonies;

placing the immobilized glucose oxidase in a sensor; and
testing the sensor.

36. (Withdrawn) A method for formulating an enzyme according to claim 35, wherein extracting glucose oxidase from the colonies comprises employing an ionic column to extract glucose oxidase from the colonies.

37. (Withdrawn) A method for formulating an enzyme according to claim 35, wherein extracting glucose oxidase from the colonies comprises:

removing the glucose oxidase from the colonies;

purifying the glucose oxidase; and

characterizing the glucose oxidase.

38. (Withdrawn) A method for formulating an enzyme according to claim 37, wherein removing the glucose oxidase from the colonies comprises grinding the colonies in a homogenizer into cell components.

39. (Withdrawn) A method for formulating an enzyme according to claim 38, wherein removing the glucose oxidase from the colonies further comprises fractionating the cell components employing centrifugation and differential solubility after grinding the colonies in a homogenizer.

40. (Withdrawn) A method for formulating an enzyme according to claim 37, wherein removing the glucose oxidase from the colonies comprises disrupting the colonies into cell components via sonication.

41. (Withdrawn) A method for formulating an enzyme according to claim 40, wherein removing the glucose oxidase from the colonies further comprises fractionating the cell components employing centrifugation and differential solubility after disrupting the colonies via sonication.

42. (Withdrawn) A method for formulating an enzyme according to claim 37, wherein purifying the glucose oxidase comprises purifying the glucose oxidase by employing chromatography methods.

43. (Withdrawn) An enzyme formulated according to the method of claim 26.

44. (Original) The method of formulating an enzyme according to claim 1, wherein the host is a host organism.

45. (Original) The method of formulating an enzyme according to claim 1, wherein determining whether the colonies contain active glucose oxidase further comprises isolating the glucose oxidase.

46. (Currently Amended) The method of formulating an enzyme according to claim 1, wherein screening the colonies for desirable properties further comprises:

isolating the glucose oxidase;

placing the glucose oxidase in a sensor; and

testing the sensor.

47. (Original) The method of formulating an enzyme according to claim 46, wherein testing the sensor comprises introducing the sensor into an accelerated test environment.

48. (Withdrawn) A method for making a biosensor comprising:
obtaining a library of glucose oxidase genes;
creating a library of mutated glucose oxidase genes;
introducing each mutated glucose oxidase gene of the library into separate expression vectors;
inserting the expression vectors into a host;
growing colonies of the host;
screening the colonies for desirable properties by determining whether the colonies contain active glucose oxidase and determining whether the colonies have peroxide resistant properties; and
placing a glucose oxidase gene having desirable properties into a sensor,

wherein determining whether the colonies have peroxide resistant properties comprises:
incubating the colonies in peroxide; and
determining whether the colonies have active glucose oxidase after incubating the colonies in peroxide, and
wherein determining whether the colonies contain active glucose oxidase comprises:
measuring a concentration of the glucose oxidase.

49. (Withdrawn) A method for making a biosensor according to claim 47, wherein screening the colonies for desirable properties further comprises testing glucose oxidase from the colonies for functionality.

50. (Withdrawn) A method for making a biosensor according to claim 47, wherein determining whether the colonies have active glucose oxidase comprises employing a substance that changes color in the presence of active glucose oxidase.

51. (Withdrawn) A method for making a biosensor according to claim 47, wherein determining whether the colonies have active glucose oxidase comprises employing a substance that changes color in the presence of active glucose oxidase.

52. (Withdrawn) A method for making a biosensor according to claim 51, wherein the substance is leuco-crystal-violet.

53. (Withdrawn) A method for making a biosensor according to claim 47, wherein determining whether the colonies have active glucose oxidase comprises checking for fluorescence.

54. (Withdrawn) A method for making a biosensor according to claim 47, wherein the host is a host organism.

In Vitro and *In Vivo* Degradation of Glucose Oxidase Enzyme Used for an Implantable Glucose Biosensor

T.I. VALDES, M.S. and F. MOUSSY, Ph.D.

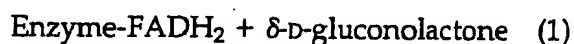
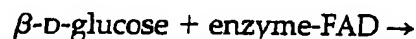
ABSTRACT

Background: The degradation of the glucose oxidase (GOD) enzyme, commonly used in the construction of glucose sensors has been of concern for scientists for decades. Many researchers have found that GOD deactivates over time, mostly due to H_2O_2 oxidation. This decay can lead to the eventual failure of the sensor. However, these findings are controversial, because other researchers did not find this degradation. **Methods:** The goal of this study was twofold. The first goal was to evaluate the *in vitro* and *in vivo* stability of two commercially available GOD enzymes and the second goal was to evaluate Nafion as a protective coating of GOD. Crosslinked GOD samples were sandwiched between two 10- μ m pore polycarbonate membranes (Nafion coated or uncoated) and placed in custom designed Lexan chambers. Chambers were then exposed to a total of five different environments: Dulbecco's Modified Eagle Medium (DMEM) or phosphate buffered saline (PBS) with and without a 5.6-mM glucose concentration, as well as the subcutaneous *in vivo* environment of 12 rats. After a period of up to 4 weeks, chambers were retrieved, opened, and tested for enzyme activity using a three-electrode system. **Results:** Enzyme activity showed only a slight decrease when exposed to DMEM and PBS without glucose. A more dramatic decrease in activity was observed in enzymes exposed to PBS and DMEM with 5.6 mM glucose. The *in vivo* environment also caused a significant decrease in enzyme activity, but the decrease was lower than for the *in vitro* environment with glucose conditions. **Conclusion:** The presence of glucose *in vitro* and *in vivo* led to the production of H_2O_2 , suggesting this to be the main agent responsible for enzyme degradation. The use of a Nafion coating did not provide any additional protection.

INTRODUCTION

GLUCOSE OXIDASE (GOD) is the most widely used enzyme in the construction of biochemical sensors.¹ There has been great interest in this enzyme because GOD has a high stability, turnover, and specificity for a commercially important analyte. GOD is a flavin adenine dinucleotide (FAD)-containing en-

zyme that catalyzes the oxidation of glucose to gluconic acid. During the catalytic cycle of GOD, the flavin prosthetic group is first reduced by glucose and then reoxidized by molecular oxygen.



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In amperometric electrochemical biosensors utilizing GOD, a subsequent reaction occurs, in which H_2O_2 is electrochemically oxidized (or O_2 is reduced) at the platinum surface of the device. From this reaction, a current is produced which is directly proportional to the concentration of glucose present in test solution.

However, many researchers found that GOD, in soluble and immobilized forms, deactivates over time, because of H_2O_2 oxidation.²⁻⁷ This decay can decrease sensor accuracy and be responsible for the eventual failure of the sensor. However, these findings are controversial because other researchers did not find degradation of GOD by H_2O_2 .⁸

Kleppe⁴ has postulated that the enzyme exists in the oxidized (FAD) and reduced (FADH_2) form. By a series of experiments, it was determined that the reduced form is inactivated by H_2O_2 at least 100 times faster than is the oxidized form. This difference remains unexplained, although a change in structural conformation is a possible cause.⁶ This difference is equivalent to saying that the deactivation is quite slow in the absence of glucose and that the oxidized form (FAD) of the free enzyme is relatively stable to H_2O_2 .⁶

Using a tubular flow reactor, Krishnaswamy⁵ also studied GOD degradation. Hydrogen peroxide was found to deactivate the enzyme severely and the deactivation rate constants were higher than those for oxygen deactivation.

The degradation mechanism of GOD is still unclear. It has been proposed, however, that the predominant mechanics for poisoning of GOD by H_2O_2 involves the attack of H_2O_2 on the glucose-GOD complex.⁶ Malikkides et al.⁶ proposed that the conformational structure of the free enzyme may change on bonding to glucose, possibly opening up the enzyme to attack by peroxide.

Kerner et al.⁹ found that glucose polarographic electrodes placed in body fluids appear to undergo a rapid decay in response over several hours and then plateau at lower sensitivity. The original response, however, is obtained when the electrode is placed back into a buffer. From these findings, Kerner⁹ hypothesized that

there is a low molecular weight material in body fluids, below 10 kDa, which interferes with the enzyme activity but that does not necessarily denature the enzyme.

Determining the exact cause of failure of a biosensor can be difficult because many events take place within the body (e.g., tissue response) and within the sensor itself (e.g., membrane degradation, poisoning, etc.) that can cause the sensor to gradually fail. Furthermore, the decline in GOD activity is also difficult to determine because glucose sensors often entrap a considerable amount of enzyme in a gel or behind a membrane.⁸⁻¹³ This masks the true rate of enzyme loss since changes in sensor performance with a reservoir of stored enzyme would not be detected unless the critical threshold of the requirement of GOD was reached.

Much research has been devoted to the GOD itself, but little of it has been directed to its function in glucose biosensors. There has also been insufficient research done on the effects that the *in vivo* environment would have on the enzyme, and previous *in vitro* experiments have not modeled the *in vivo* environment well. The goal of this work was thus to study the stability of GOD under *in vitro* and *in vivo* conditions. To be able to apply the findings of this study to glucose sensors we utilized the same GOD preparation and protective membrane used in our implantable glucose sensor. Our miniaturized amperometric glucose sensor was designed for subcutaneous implantation and is based on a tri layer membrane configuration, consisting of Nafion, GOD, and poly (*o*-phenylenediamine).¹⁴

The experiments below describe the experiments and findings regarding the degradation of GOD in a biological environment. We evaluated (1) the stability of GOD both *in vitro* and *in vivo* and (2) the efficacy of Nafion as a protective coating. Sigma (St. Louis, MO) and Biozyme (San Diego, CA) GOD enzymes were tested for stability.

MATERIALS AND METHODS

GOD preparation

GOD from two different suppliers was tested; Sigma (St. Louis, MO) (Type X-S,

DEGRADATION OF GLUCOSE OXIDASE

187,300 U/g solid, catalase impurity: 0.3 Sigma U/mg protein) and Biozyme (San Diego, CA)(363 U/mg protein, 0.016 U/mg, GO/CAT ratio: 17900). In a small glass beaker, 19.5 mg of GOD and 73.2 mg of bovine serum (Sigma) were dissolved in 1 mL of glutaraldehyde prepared solution. After stirring the suspension for 5 minutes, 50 μ L were placed on top of an 11-mm-diameter nylon mesh. This mesh was sandwiched between two 10- μ m pore polycarbonate membranes (Millipore, Bedford, MA) that had been dip-coated with Nafion or that had been left without Nafion.

These components were placed in a 13-mm-diameter Lexan chamber. Two stainless-steel screws were put in place, to hold the chamber together (Fig. 1). Lexan was the material of choice for this study, as it is a derivative of polycarbonate and can withstand high temperatures. Stainless-steel screws were used to hold the chambers together. The chambers, including the GOD and polycarbonate membranes within them, were sterilized by heat at a temperature of 120°C for 1 hour. This method of sterilization simultaneously cured the Nafion membranes, while still preserving the enzyme activity.¹⁵

Nafion-treated membranes

As a previous study showed, Nafion calcifies both *in vitro* and *in vivo*.¹⁶ The calcification of Nafion leads to degradation of the membrane with a change in its permeability to glucose. To avoid the calcification of the Nafion membranes in the culture media or in the *in vivo* environment, the polycarbonate membranes covered with Nafion were pre incubated for 24 hours in a 0.1 M FeCl₃ solution. We previously showed that this treatment prevents the calcification of Nafion.¹⁷

In vitro studies—no glucose

Chambers represented four different sample preparations: Sigma enzyme, with and without Nafion, as well as Biozyme enzyme with and without Nafion. Each of the sample preparations was exposed to two different kinds of *in vitro* environment. The chambers were placed in heat sterilized flasks with sterile Dulbecco's Modified Eagle Medium (DMEM) or phos-

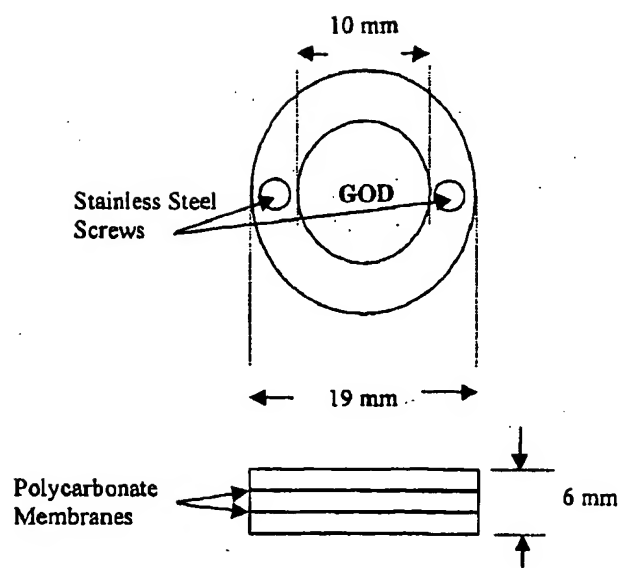


FIG. 1. Schematic of a Lexan chamber used for the isolation of glucose oxidase held together by stainless-steel screws. The inside contains the GOD enzyme, protected by polycarbonate membranes coated with Nafion or left without it.

phate buffer saline (PBS) for up to 4 weeks (at 37°C). DMEM is used for cell culture experiments. It contains peptides, vitamins and amino acids, and in past experiments, has served as a good model for *in vivo* experiments.^{16,17} There was no glucose in the DMEM or PBS solutions. Six chambers were prepared per condition per week and the chambers were then tested at Weeks 1, 2, and 4. The goal of this study was to determine whether compounds other than H₂O₂ would degrade the enzyme *in vitro*. Because no glucose was present, there was no production of H₂O₂ by the enzyme.

In vitro studies with 5.6-mM glucose solution

To discern whether degradation of the enzyme would occur in the presence of the by-product H₂O₂, chambers containing either Sigma or Biozyme enzyme were placed in DMEM or PBS containing a 5.6-mM concentration of glucose (at 37°C) for up to 4 weeks. The polycarbonate membranes used for this experiment were all coated with Nafion, to slow the diffusion of H₂O₂ out of the chamber, and thus induce a significant H₂O₂ concentration close to the enzyme, as in the case of a glucose sensor. If in fact, the H₂O₂ produced de-

graded the enzyme, the effect would have been better detected using Nafion-coated polycarbonate membranes because without Nafion, H_2O_2 would easily diffuse into the bulk of the solution.

In vivo studies

Twelve Sprague-Dawley rats were implanted subcutaneously with four GOD chambers each, representing the four different sample preparations, as described above (Sigma/Biozyme, with or without Nafion). Anesthesia was accomplished by initially placing the rat in an induction chamber filled with isoflurane. It was then maintained throughout the surgery using a continuous-flow gas anesthesia system. Using aseptic technique, two three-inch incisions were made on the back of each of the rats. Two chambers were placed toward the anterior end, and two toward the posterior end. Implants were not anchored. Following implantation, incisions were closed using surgical staples. Rats were monitored closely after surgery for signs of clinical distress, such as lack of grooming, anorexia, weight loss, or diarrhea. The chambers were recovered from four rats for Weeks 1, 2 and 4, and GOD in the chambers were tested as described below.

GOD and glucose concentration measurements

At Weeks 1, 2, and 4, the chambers were removed from their respective solutions or from the *in vivo* environment. To measure the H_2O_2 production, and thus the activity of the enzyme, the chambers were opened and the top polycarbonate membranes removed. The removal of the top membrane eliminated the diffusion barrier to glucose of the membrane. This ensured an accurate current reading, which corresponded to the true activity of the enzyme. The opened chambers were then preincubated in a warm water bath at 37°C in PBS for one half hour. The GOD activity was then measured using a three-electrode system. A counter and calomel electrodes as well as a pure Pt Wire (99.5% Pt) were submerged in 50 mL of PBS kept at 37°C . The solution was constantly stirred at 120 RPM using the Labo-Stirrer (Yamato Scientific, Tokyo, Japan). The Pt

wire was powered with +0.7 Volts using the CV-27 potentiostat (Bioanalytical Systems, Inc., West Lafayette, IN,). Once stabilization of the response current was confirmed, 3 mL of a 0.1-M glucose solution was added to make a 5.6-mM test solution. Prior to utilization, the glucose solution was allowed to mutarotate (from α to β anomer) overnight because the enzyme is highly specific for β -D-Glucose. It was then allowed to react with the GOD and the resulting oxidizing current was recorded.

Statistical analysis

To test for significant differences ($p < 0.05$) between the results, we used the one-way analysis of variance test (ANOVA). Because our data had a normal distribution and unrelated samples, this was thought to be the most suitable test. ANOVA compares the amount of heterogeneity *within* samples with the amount *between* samples. Because all samples were tested the same way, with reproducible results, the data being analyzed were examined for only one parameter, namely, whether or not there was a difference in enzyme activity.

RESULTS

Reproducibility of GOD sample preparation

We had to verify that the preparation of the GOD samples yielded the same results each time a new batch was made. To maintain the same results, each batch was allowed to cross-link for the same amount of time (5 minutes) before being placed in the GOD chambers. To test for this, two different batches were made, and tested for their response, without any prior incubation in any solution. The data proved to be almost identical (data not shown). The consistency of GOD sample preparation was repeatedly proved throughout this study because all batches had similar initial activities in response to glucose.

Measurement accuracy

We also had to verify that the size (i.e., surface area) of the working electrode (Pt wire) would be sufficient to detect the H_2O_2 pro-

DEGRADATION OF GLUCOSE OXIDASE

duced by the enzyme in a linear fashion and over the expected concentration range. Figure 2 represents a calibration standard, which asserts the functionality of the three-electrode system, described above. This curve was made using several dilutions of 30% H_2O_2 (Sigma). As can be discerned from the curve, the system is quite sensitive to changes in H_2O_2 concentrations. The data obtained from the GOD chambers (with a maximum of 1600 nA) is well within the range observed in the calibration curve.

Each of the GOD samples, whether implanted or kept in *in vitro* conditions, was tested using the three-electrode system described above. The Pt electrode was powered with +0.7 V and therefore allowed the produced H_2O_2 to be oxidized at its surface. Because the surface area of the wire immersed in the solution is directly proportional to the amount of H_2O_2 oxidized at its surface, great care was taken as to maintain the same surface area each time a test was performed.

In vitro—no glucose

Figures 3 and 4 are bar graphs (mean \pm SD, $N = 6$) of the current produced by GOD chambers after incubation in PBS and DMEM with-

out glucose for a period of up to 4 weeks. Week 0 represents the initial values before exposure to solution. We notice from these graphs a limited decrease in response current in these different environments that appears as early as Week 1 and remains stable until Week 4. In addition, there is no statistically significant difference between Sigma GOD and Biozyme GOD. There is also no statistically significant difference between the enzymes that were protected with Nafion and those that had no Nafion protection.

More importantly, there is also no visible difference between chambers that were preincubated in DMEM in comparison with those in PBS. We believe that the peptides present in DMEM did not cause fouling of the enzyme. Also, because there was no glucose present in this experiment, there was no production of H_2O_2 . The slight decrease in enzyme activity that was observed in PBS and DMEM could have been caused by elution of poorly trapped GOD.

In vitro with 5.6-mM glucose solution

In the presence of a 5.6-mM glucose solution, whether in PBS or DMEM, the chemical reaction previously described which produces

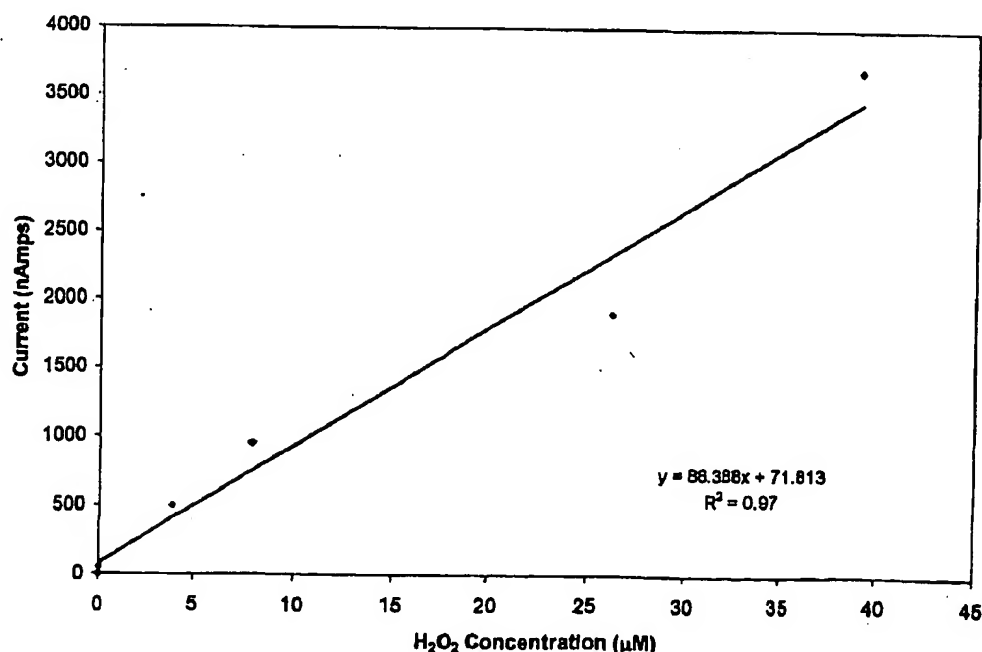


FIG. 2. Three-electrode system calibration curve.

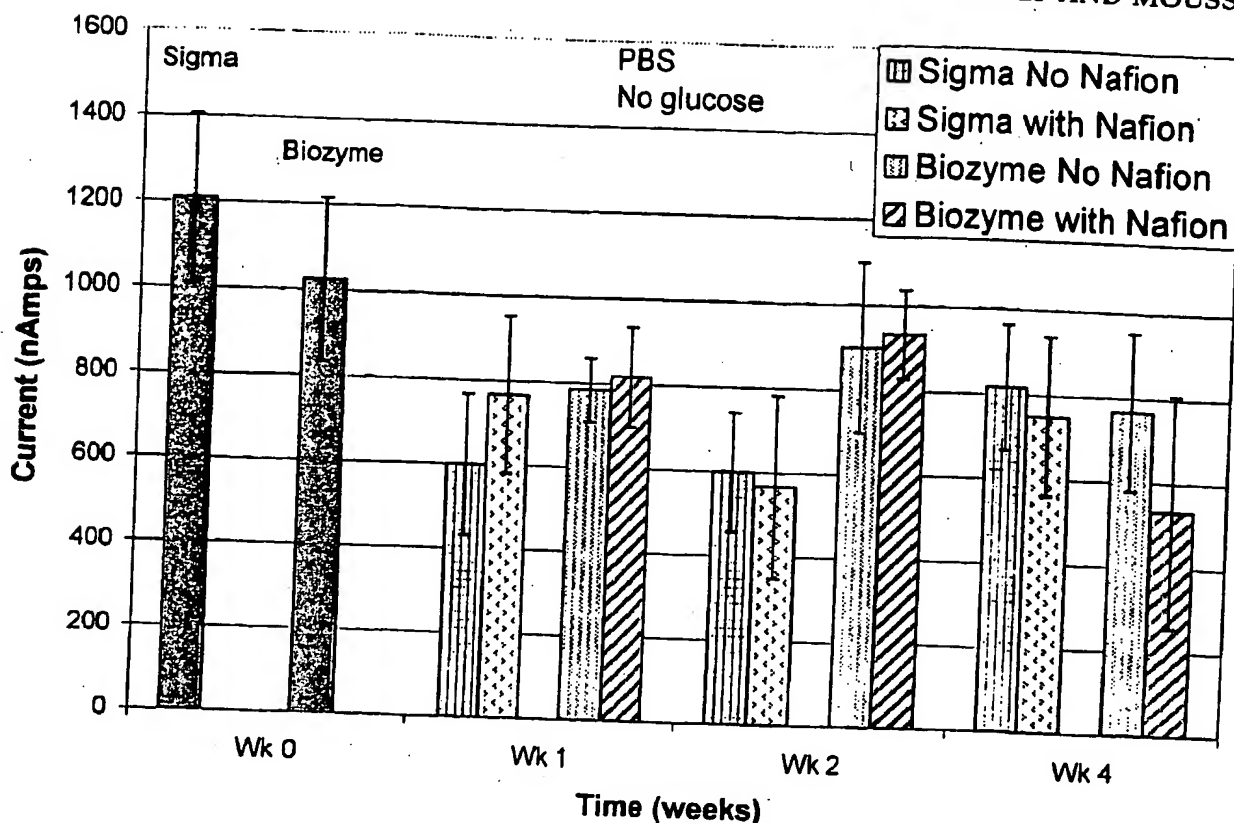


FIG. 3. *In vitro* testing of GOD chambers with or without Nafion after exposure to PBS. There was no glucose in the solution. Week 0 represents the initial values before exposure to solution (Mean \pm SD, $n = 6$).

H₂O₂ as its by-product, was allowed to take place inside the solution. H₂O₂ has been suspected to denature the GOD enzyme over time.²⁻⁷ As discerned in Figure 5, there is a gradual decrease in the response current. Week 4 shows severely reduced enzyme activity, thus confirming the degradation of enzymes by H₂O₂ ($p < 0.05$ for comparisons between Week 4 versus Week 0 for Sigma and Biozyme, ANOVA). No difference was observed between enzymes obtained from Sigma and Biozyme. Finally, the degradation appears to be more severe in DMEM than in PBS at Week 4 ($p < 0.05$, ANOVA). The reason for this effect is unclear because it was not observed with the *in vitro* results in the absence of glucose.

In vivo

After retrieval, the GOD enzyme chambers were tested using the three-electrode system described above. The currents corresponding to each of the different weeks were plotted, and their error bars calculated as standard deviation

($n = 4$). Figure 6 shows a significant decrease in current ($p < 0.05$ for all comparisons between Weeks 1, 2, and 4 versus Week 0 for both Sigma and Biozyme, ANOVA) following exposure to the *in vivo* environment. The greatest drop in current occurred in the Sigma GOD enzyme (compared to the Biozyme product), perhaps suggesting this to be the least stable of the two enzymes tested. Half of the membranes tested had polycarbonate membranes coated with Nafion. However, Nafion did not prove to make a significant difference in protecting the GOD enzyme because there were only slight differences in the response of chambers coated with Nafion. This is consistent with the theory that H₂O₂ is the primary source of enzyme degradation.

DISCUSSION

By testing the activity of GOD following *in vitro* incubation in the presence or without glu-

DEGRADATION OF GLUCOSE OXIDASE

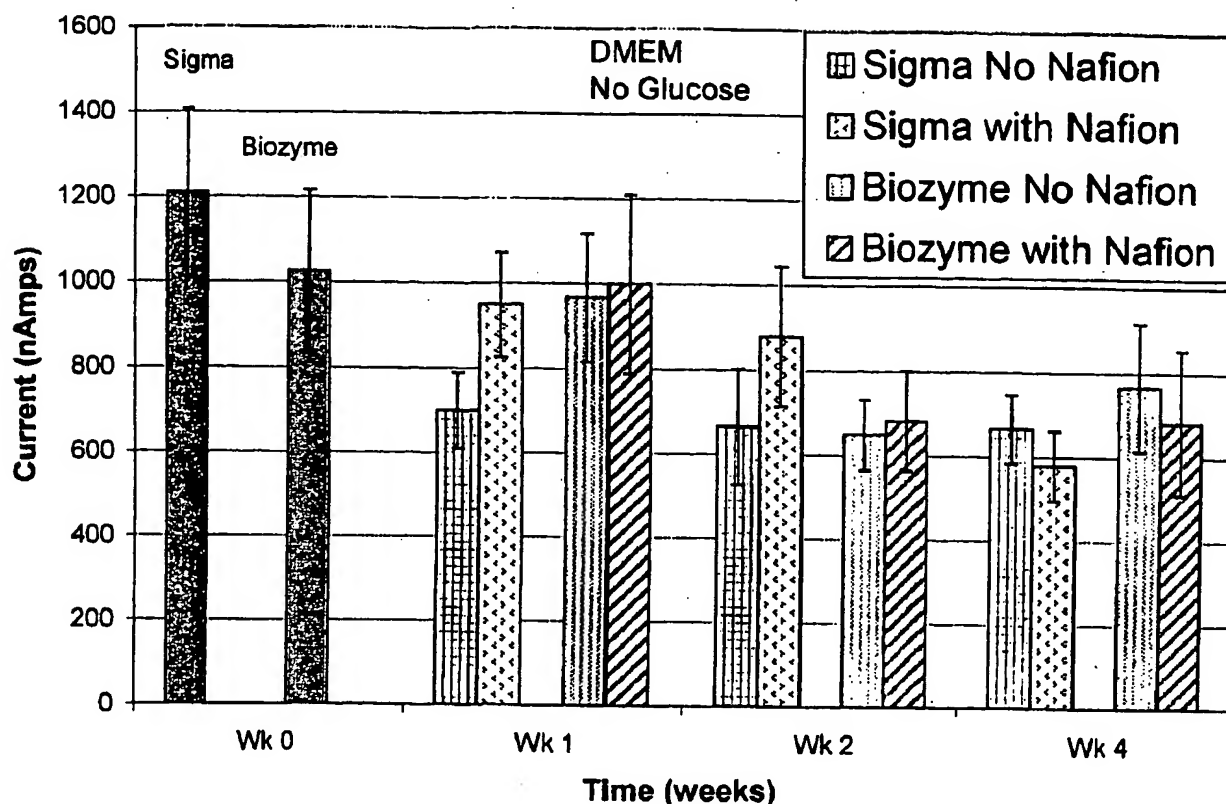


FIG. 4. *In vitro* testing of GOD chambers with and without Nafion after exposure to DMEM. There was no glucose in the solution. Week 0 represents the initial values before exposure to solution (Mean \pm SD, $n = 6$).

cose, we observed a rapid degradation of the enzyme in the presence of H_2O_2 that was generated by the oxidation of glucose. Similarly to the *in vitro* results in PBS and DMEM with 5.6 mM glucose, there was a significant decrease in enzyme function in the *in vivo* environment. However, the decrease in response observed after implantation was less than when the chambers were incubated in PBS or DMEM with 5.6 mM glucose. It is possible that during incubation, H_2O_2 concentration in the chambers was lower under *in vivo* conditions than in the *in vitro* environment because H_2O_2 could have been physiologically oxidized. The lower H_2O_2 concentration would therefore result in less degradation of the enzyme. In contrast, in the *in vitro* studies the six chambers were placed in 50 mL of 5.6 mM PBS or DMEM, and the continuous production of H_2O_2 in this fixed volume could have led to increasing H_2O_2 concentration and thus exacerbated the decrease in enzyme function.

Another hypothesis as to why the decrease

was less *in vivo* than *in vitro* is that in the *in vivo* environment, the local concentration of glucose and also O_2 , could have been lower than *in vitro* (e.g., as a result of the tissue reaction to the implant), therefore leading to lower reaction and thus to lower hydrogen peroxide production. It is speculated that inflammation could have consumed a significant amount of glucose and oxygen and that fibrous encapsulation could have provided a diffusion barrier to glucose (and perhaps oxygen), thus decreasing the amount of analyte in the chamber. It is also possible that an unknown variable in the body may actually help to preserve the GOD.^{7,9}

Furthermore, because the GOD enzyme chambers stimulated the typical host responses to a foreign object, it was thought that secretion products from inflammatory cells could have lead to the rapid degradation of the enzyme. However, this did not appear to be the case. The enzymes that were protected only by the 10- μ m pore polycarbonate membranes were not more degraded than the enzyme pro-

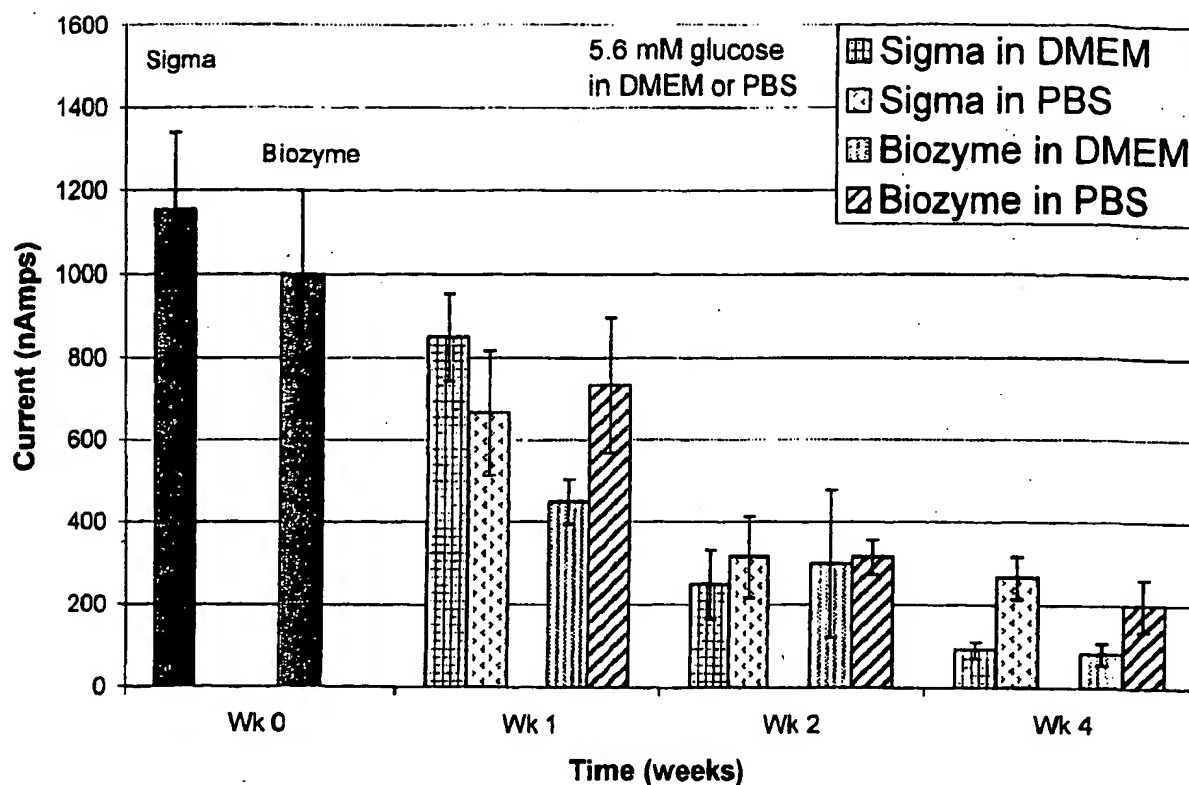


FIG. 5. *In vitro* testing of GOD chambers after exposure to 5.6-mM glucose DMEM and PBS. Week 0 represents the initial values before exposure to solution. All GOD samples were protected with polycarbonate membranes and Nafion (Mean \pm SD, $n = 6$).

ected by polycarbonate membranes coated with Nafion. This observation confirms the hypothesis that H_2O_2 production is the main cause for GOD degradation, and suggests that the polycarbonate membranes provided some protection to the enzyme, perhaps by restricting cell access to the enzyme. It is likely that if the inflammatory cells (such as macrophages) could have readily migrated toward the enzyme that they would have degraded it rapidly.

Application to a glucose sensor

This study demonstrated the progressive degradation of GOD both *in vitro* and *in vivo* over a period of 4 weeks under the experimental conditions described in this paper. H_2O_2 production was shown to be the main cause of degradation. However, this effect may not be as significant in a glucose sensor such as our miniaturized sensor.¹⁴ Indeed, the local concentration of H_2O_2 encountered in these ex-

periments is most likely higher than the concentration in a miniaturized glucose sensor because the amount of enzyme and the geometry (i.e., surface area of the membranes) are different. Unfortunately, it is difficult to precisely measure H_2O_2 concentration in the enzyme layer in the closed chamber as well as in a miniaturized glucose sensor such as ours. Nevertheless, this study reemphasizes that GOD-based sensors should utilize external membranes that are not too permeable to glucose to reduce the amount of H_2O_2 produced.

Furthermore, our glucose sensor has an excess of enzyme, and even very low remaining enzyme activities are suspected to be more than what is required to catalyze the incoming glucose. If however, the lifetime of the sensor was threatened because of enzyme degradation, several steps could be taken to ensure longer sensor functionality.

The sensor could be loaded with the enzyme to an even higher extent. Another option, could be the replacement of the degraded enzyme by

DEGRADATION OF GLUCOSE OXIDASE

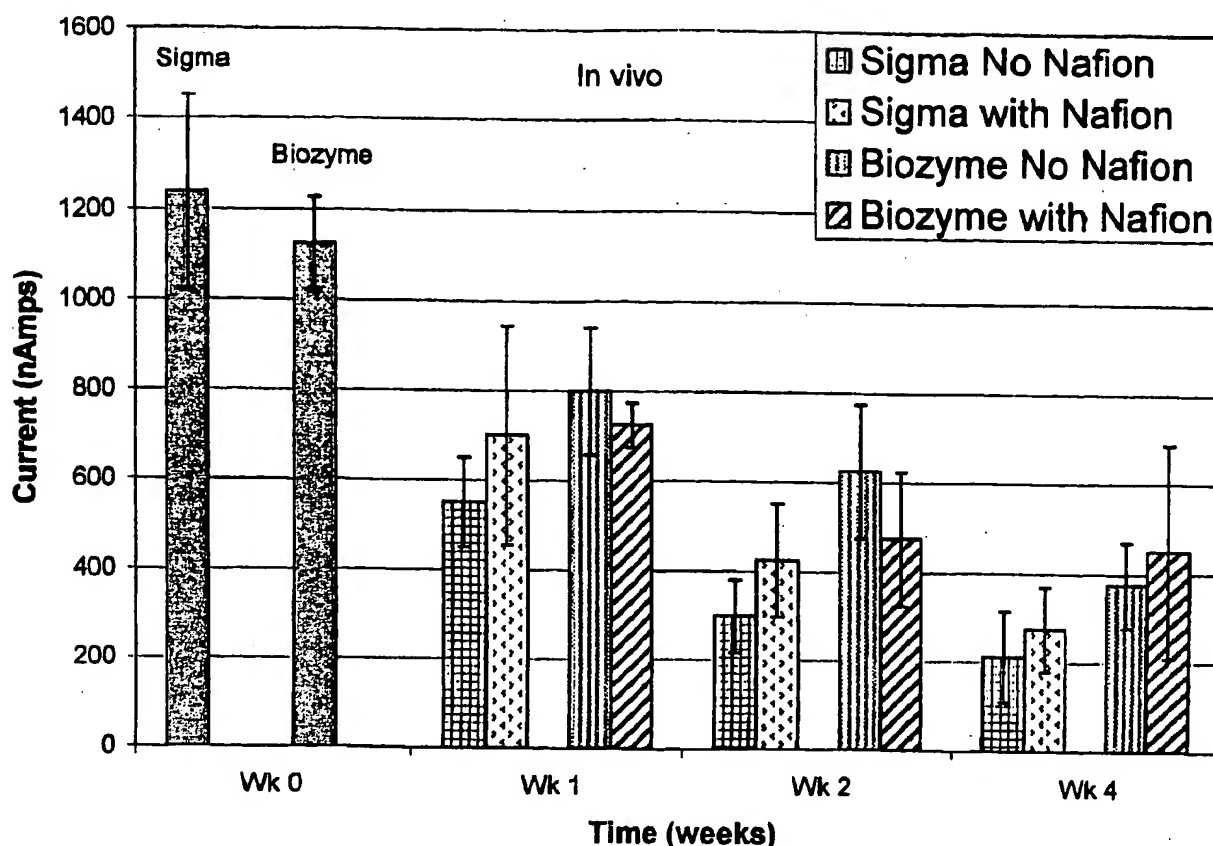


FIG. 6. *In vivo* testing of glucose oxidase chambers. Week 0 represents the initial values before implantation (Mean \pm SD, $n = 4$).

fresh enzyme, similar to the approach used by Wilkins' group.¹⁸ Better options, however, are to simply prevent the degradation of the enzyme using other chemical agents, or techniques.

To prohibit the H_2O_2 from degrading the GOD enzyme, it has been proposed that catalase be coimmobilized with GOD because catalase is the natural hydrogen peroxide destroying catalyst.¹⁹ The addition of catalase in either the GOD itself, or to the incubating solution has resulted in a slower deactivation of the GOD enzyme.^{7,20,21} However, this solution is really more appropriate for glucose sensors based on the detection of O_2 that do not depend on measuring H_2O_2 . Furthermore, catalase is in turn inactivated by hydrogen peroxide.^{6,19,22}

A long-term remedy of the degradation of GOD by H_2O_2 could be the immobilization and attachment of the enzyme to a support that deactivates H_2O_2 , as it is being produced. Such a study was conducted by Cho,² using the per-

oxide decomposition catalyst, activated carbon. In a study conducted by Carter,¹⁹ the best results were obtained with activated carbon, impregnated with ruthenium. This combination was able to destroy hydrogen peroxide and stabilized the enzyme.

In conclusion, this paper demonstrates that H_2O_2 is the main cause for degradation of GOD *in vitro* and *in vivo*. Furthermore, the use of a Nafion membrane did not provide any additional protection for the enzyme (as compared with the enzyme protected by just a 10- μ m pore polycarbonate membrane) even *in vivo*. Further research is therefore needed to make GOD less sensitive to H_2O_2 degradation, especially for long-term implantable glucose sensors.

ACKNOWLEDGEMENT

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Kinetic Assay Methods

The design and use of a particular enzyme assay is dependent on the objectives of the particular investigation. If the focus is on enzyme purification or issues of clinical biochemistry, experimental protocols typically require optimizing conditions for all substrates and effectors—e.g., with respect to pH value, temperature, buffering agent, and ionic strength. However, optimal conditions may not be reasonable for all parameters and factors. For example, substrate instability may preclude studies at the enzyme's optimal pH or at the physiological temperature. Thus, Silverstein and Sulebele (1969) examined the exchange rates of malate dehydrogenase at 1°C to avoid problems arising from the decomposition of oxaloacetate. Likewise, in clinical studies and enzyme purification, saturating concentrations of substrate(s) are typically required, although this may not be possible or even desirable in many instances, as limitations in substrate solubility or substrate inhibition of the enzyme may discourage the use of elevated concentrations. The focus in clinical studies and enzyme purification is on providing good estimates of the activities of a specific enzyme in a number of physiological samples with a rapid, straightforward, and reproducible assay method. However, for the kineticist the objective is to characterize the initial rates of the enzyme-catalyzed reaction under a wide set of conditions. It is through these detailed studies, often carried out at subsaturating substrate concentrations, that the investigator is able to determine the true optimal parameters to be used in protein purification and clinical studies, as well as to probe the dynamics and mechanism of the reaction and its modulation by other factors. The purpose of this appendix is to provide a brief review of issues important in the design of initial-rate assay methods.

In most instances, the investigator already has some information about the kinetic properties of the enzyme, obtained from preliminary experiments as well as from the literature. It is necessary to have an approximation of the magnitude of the Michaelis constants of all the substrates in order that detailed initial rate studies can be effectively designed. Certainly, information on the identity of the substrates and products is required, along with an effective procedure for monitoring substrate depletion or product accumulation. The nature of the reac-

tion and the properties of the substrates help dictate the assay procedure used. Enzyme-assay methods include those based on spectrophotometry, radiometry, pH-stat, turbidometry, polarography, and fluorometry. Spectrophotometric and fluorometric assay protocols rely on the differences in spectral or fluorescent properties, respectively, of the product(s) relative to the substrate(s). With radiometric procedures, effective means for separating labeled substrate(s) from labeled product(s) must be available so that the amount of product formed at a given time can be accurately measured. Those reactions that utilize or generate protons (or hydrons) can be followed by pH-stat methods. If the reaction studied results in a change in solution turbidity (e.g., caused by aggregation of a macromolecular complex), then turbidometric protocols can effectively assay for the protein that causes the change in turbidity. All of these methods, as well as others, can be found in Bergmeyer (1983).

GENERAL ASPECTS OF ASSAY DESIGN

An enzyme's progress curve (i.e., a plot of product formation or substrate depletion as a function of time; see Fig. A.3H.1) consists of four phases that are of interest to the investigator. The duration of the **pre-steady-state** region is relatively short and is typically followed via rapid-flow or relaxation techniques. Discussion of this region of the progress curve is beyond the scope of this appendix, but a review of these techniques has recently appeared (Fierke and Hammes, 1995). The next portion of the curve is the **steady-state** phase, a longer phase characterized by relatively constant concentrations of each of the individual enzyme forms (e.g., the enzyme-substrate binary complexes). During the third phase of the progress curve—the **post-steady-state** region—the levels of these enzyme complexes change rapidly, until the last phase—**equilibrium**—has been reached. Each of these phases contains information of use to the investigator. The steady-state portion of the curve typically exists for a few to several hundred seconds. It is this region of the curve that is most accessible to the majority of investigators. In addition, the relatively straightforward mathematical treatment of steady-state kinetics provides an excellent foundation for the subsequent characterization

APPENDIX 3H

Commonly Used Techniques

A.3H.1

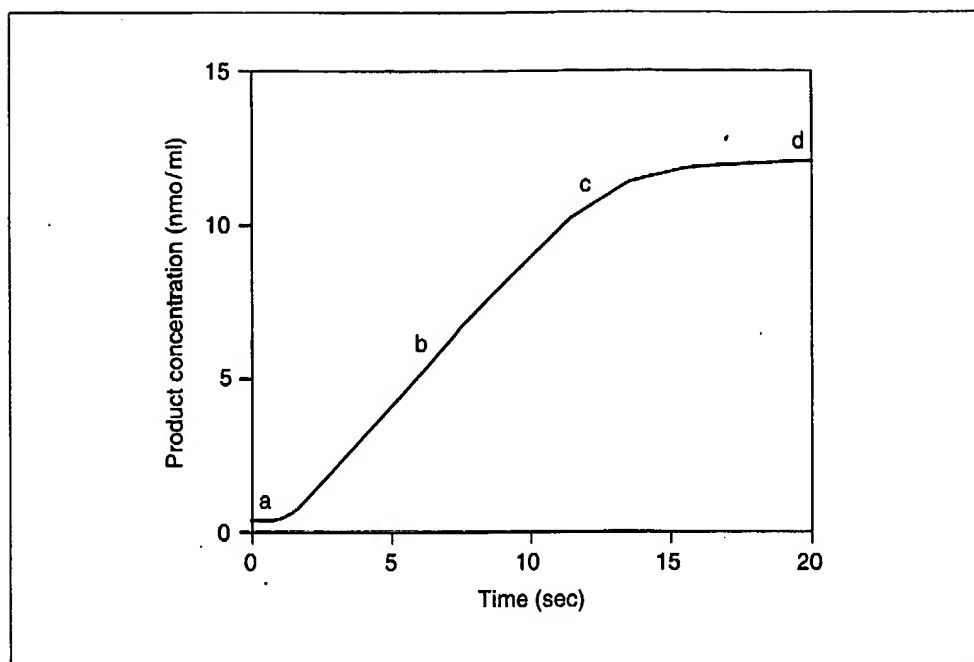


Figure A.3H.1 Typical progress curve for an enzyme-catalyzed reaction, where *a* represents the pre-steady-state region, *b* the steady-state region, *c* the post-steady-state region, and *d* the equilibrium region.

of the enzyme system. Because enzyme rate expressions can be quite complex, a number of assumptions are made to linearize the equations. The investigator should ensure that each of these factors are addressed when any assay protocol is being designed.

Maintaining Constant Reaction Conditions

The status of each experimental parameter—e.g., temperature, ionic strength, and pH—should remain constant over the time course of the assay.

Temperature

Prior to initiation of the reaction by addition of a small aliquot of enzyme or substrate, the reaction mixture should be thermally equilibrated with an accurately controlled constant-temperature water bath for several minutes. If the substrate is thermally stable, this incubation should be for ≥ 10 min or until the desired reaction temperature is reached. If the substrate is unstable at this temperature, it may be necessary either to assay at a different temperature, to correct for substrate decay with time, or to initiate the reaction by addition of a small aliquot of substrate solution that was freshly prepared and maintained in an ice bath. When the reaction is initiated by addition of either the enzyme or the substrate, the volume of the

aliquot should be small enough to minimize any alterations in temperature upon addition. Typically, there is at least a hundred-fold difference in volume between the reaction mixture and the initiating aliquot. If the enzyme and substrate are stable under the assay conditions, then both solutions should be preincubated at this temperature.

It should be emphasized that valid initial rate experiments are only obtained when good temperature control is maintained. The temperature-regulated water bath should have a variability of $\leq 0.1^\circ\text{C}$. When the reaction is initiated, the solution should be thoroughly mixed. If reaction progress is followed using an instrument such as a spectrophotometer, the sample compartment should be thermally isolated.

Another issue of concern is choice of the temperature at which enzyme activity will be measured. In 1964, the International Union of Biochemistry (IUB) recommended a temperature of 30°C . Clinical chemists have often suggested a change to 37°C . However, the final choice of temperature will be dependent on the system being investigated. For example, the activity of glutamate dehydrogenase isolated from the hyperthermophile *Pyrococcus furiosus* is negligible at both 30°C and 37°C ; the enzyme exhibits maximal activity at 97°C (Klump et al., 1992). Clearly, at some point in the investigation, the effect of temperature on

the catalytic activity will have to be characterized.

Buffer selection

In setting up the assay protocol, care should also be exercised in selecting an appropriate buffer agent. Unfortunately, many investigators choose a buffer solely on the basis of the desired pH of the reaction medium. Many excellent buffers, however, have a significant oxidation-reduction potential that can severely alter an enzyme's activity. For example, cacodylate ($pK_a = 6.27$) and other organoarsenicals have a potent oxidizing potential under acidic conditions. Borate ($pK_a = 9.23$) and other borate-based buffers form complexes with many diols, polyols, carbohydrates, and ribonucleotides; for example, in the presence of borate ion, both L- and D-serine form a transition complex and strongly inhibit γ -glutamyl transpeptidase (Tate and Meister, 1978). Hence, care should be exercised in selecting a buffer.

In determining which buffer is optimal for a given enzyme system, it is best to examine the enzyme's activity with as many buffers as possible. Buffers in whose presence the enzyme exhibits high activity should then be examined to determine if the buffering agent interacts with the protein. If pH and ionic strength are held constant, the enzyme activity should be independent of the buffer concentration. Another factor of importance in selecting the appropriate buffer is the capacity of the buffering agent to bind metal ions. Ideally, a good buffer will not form any significant complexes with metal-ion cofactors.

Metal-ion concentrations

The role of metal ions is crucial with many enzymes, particularly with systems that require nucleotides—e.g., phosphotransferases. Care must be exercised in designing initial-rate assays to ensure that the concentration of free metal ions does not vary significantly with substrate concentration. Thus, when varying the concentrations of substrates such as ATP, the concentration of free, uncomplexed Mg^{2+} should remain relatively constant. One of two methods is commonly utilized to achieve this end: either (1) maintaining the total nucleotide concentration and the metal concentration at a constant stoichiometric ratio (e.g., 10:1) or (2) maintaining a constant excess of metal ion over the total nucleotide concentration (e.g., 1.0 mM free Mg^{2+}). The second method, which has been demonstrated to be the preferable of the two (O'Sullivan and Smithers, 1979), requires

knowledge of the stability constant for the metal-ion-nucleotide complex under the conditions of the assay.

Substrate Stability and Purity

It is crucial to establish the degree of stability of the substrate as well as that of any enzyme effector under the assay conditions (also see discussion of Maintaining Constant Reaction Conditions). Substrate and effector stabilities can be tested by preparing a solution of the compound at a concentration that will be used experimentally with the other components of the assay mixture, but without the enzyme. Then, samples are removed at different periods of time, usually over a period of several hours, and assayed either chemically or enzymatically (Bergmeyer, 1983). A plot of concentration as a function of time will reveal if the substrate is stable or if there is a decay. When present, this decomposition is usually a first-order process and can be described by the equation $[S]_t = [S]_0 e^{-kt}$, where $[S]_t$ and $[S]_0$ are the substrate concentrations at time t and time 0, respectively, and k is the first-order rate constant. If the decomposition is greater than first-order or composed of more than one first-order process, then the decay must be evaluated by the appropriate expression, which can be found in any basic kinetics text.

An issue related to substrate stability is substrate purity. Impurities in the substrate, effectors, or enzyme preparation can be potential sources of experimental error. Because alternative substrates and competitive inhibitors are often structurally similar to the substrate and frequently have similar chemical and physical properties, it is not surprising to find these compounds as impurities in commercial substrate preparations. Spectral and chromatographic analyses, as well as enzymatic analysis, are crucial in establishing the purity of a particular biochemical substance. If impurities are present in a reaction mixture, a kinetic analysis will give wrong estimates of the kinetic parameters (Allison and Purich, 1979). Heavy-metal ions are often found as impurities in commercial substrates. To remove these, it may be necessary to recrystallize the substrates, possibly in the presence of a chelating agent such as EDTA.

These issues of purity and stability are also pertinent for any modulators of enzyme activity, including the product(s). The products have to be stable over the time course of the assay. In some instances, the precise nature of the true product of the enzyme-catalyzed reaction may

not be known. In these cases, the investigator may follow the reaction via substrate depletion. It is frequently useful to do a kinetic analysis of freshly prepared substrate in the absence and presence of enzymatically prepared product. A decent understanding of the possible presence of product inhibition in initial-rate experiments is always useful to the protein chemist.

Enzyme Stability and Purity

Enzymes often undergo a slow inactivation. Usually, the rate of this inactivation is accelerated by dilution of the stock enzyme preparation. Efforts have to be made to eliminate or lessen the loss of enzyme activity with time. Often, multisubstrate enzymes are stabilized by the presence of a substrate. For example, tubulin:tyrosine ligase is stabilized by the presence of its protein substrate (Deans et al., 1992) and the presence of Mg^{2+} -ATP is known to stabilize a number of phosphotransferases. If the enzyme inactivation can be retarded by the presence of a substrate, the investigator should note the amount of that substrate that is present with the enzyme so that proper corrections can be made when the reaction is initiated with the full assay mixture.

Other agents—e.g., glycerol, certain salts, high-ionic-strength solutions, and EDTA—have also been used to stabilize proteins. Often, inactivation is a result of surface denaturation or adsorption of the enzyme on glass surfaces following dilution. The presence of stabilizing agents and/or use of plastic containers can lessen these effects significantly. The presence of serum albumin or another protein will frequently minimize the effects of enzyme dilution; the investigator should test several proteins when this technique is used. The stabilizing protein may also bind the substrates or the enzyme's cofactors; if this is the case, corrections have to be made.

If a stabilizing agent cannot be identified, the inactivation should be minimized by using the smallest dilution possible. If practical, the reaction may be initiated by the addition of one of the substrates. Corrections for loss of enzyme activity can also be made by establishing an activity-decay curve. However, it should be verified that the loss of activity is due to total inactivation and not simply to a conformational change to a form having less activity (i.e., a form with different kinetic parameters). This possibility could be tested by kinetic assays at significantly different times following dilution or by two reference assays, one using a saturat-

ing substrate level and the other using a subsaturating substrate level.

The presence of thiol reagents—e.g., 2-mercaptoethanol, dithiothreitol, or dithioerythritol—is routinely helpful in maintaining activity for those enzymes that require reduced thiol groups for activity. Care must be exercised that the reducing agent does not produce other effects. For example, γ -glutamylcysteine synthetase is inhibited by the presence of thiol reagents even though cysteine is a substrate (Seelig and Meister, 1985).

Enzyme purity is also an issue of concern. It is important in early initial-rate studies to eliminate contaminating activities and to demonstrate that any minor activity does not substantially affect the reaction being studied. For multisubstrate enzymes, this can be readily accomplished by observing the stability of each substrate, effector, and product separately in the presence of the enzyme preparation. Adenylate kinase is a common contaminant in preparations of a wide variety of phosphotransferases and other nucleotide-dependent enzymes. This contaminating enzyme can be inhibited by performing the assay at high ionic strength (Bowen and Kerwin, 1955). Addition of elevated levels of AMP will also inhibit adenylate kinase. However, many phosphotransferases are also inhibited by this mononucleotide or are themselves inhibited by high ionic strength. Purich and Fromm (1972) and Lienhard and Secemski (1973) have shown that the multisubstrate analogs P^1, P^4 -di(adenosine-5')-tetraphosphate and P^1, P^5 -di(adenosine-5')-pentaphosphate are potent and specific inhibitors of this activity.

In some instances it may be impossible to completely eliminate side reactions. Enzymes that have branched reaction pathways (i.e., multifunctional enzymes) will exhibit two pathways. Most multifunctional enzymes are transferases having multiple acceptor specificity. Glucose-6-phosphatase catalyzes not only the hydrolysis of glucose-6-phosphate but also its synthesis; thus, it may effect the transfer of phosphoryl groups from pyrophosphate, carbamoyl phosphate, or other glucose-6-phosphate molecules (Nordlie, 1982). This synthetic role of glucose-6-phosphatase is not a minor activity; at a concentration of 100 mM D-glucose, the synthesis and hydrolysis activities are approximately equal. The enzyme γ -glutamyl transpeptidase catalyzes the hydrolysis of a number of γ -glutamyl-containing molecules (including glutathione and glutathione disulfide) and also exhibits a transferase activ-

ity in which the glutamyl moiety is transferred to any of a number of amino acid acceptors, particularly cysteine, glutamine, and methionine (Allison, 1985). The hydrolase and transferase activities of γ -glutamyl transpeptidase are approximately equal in the presence of a pool of acceptor substrates at their physiological concentrations (Allison and Meister, 1981). Asparagine synthetase catalyzes the glutamine-dependent synthesis of L-asparagine and also exhibits a significant glutaminase activity (Boehlein et al., 1994). In all of these cases, it is possible to use inactivation studies to observe the parallel loss of both (or all) activities with time.

Enzyme Concentration

The duration of the steady-state phase of an enzyme-catalyzed reaction is dependent on a number of factors. Perhaps the most important of these is the relative concentration of the enzyme and substrate. The ratio of the initial substrate concentration to the total enzyme concentration should be $\geq 100:1$. With lower values, the steady-state phase will be of very short duration. It should be pointed out that this relationship holds for enzyme effectors as well. Thus, when investigating the influences of an activator or competitive inhibitor on enzyme activity, the total enzyme concentration should be much less than the concentration of the effector. This may prove difficult if the effector has a very tight affinity with the enzyme. In these cases, the protocols outlined by Williams and Morrison (1979) will prove useful.

Initial Rate and Steady-State Condition

In an experiment, the investigator typically measures the rate of an enzyme-catalyzed reaction by calculating the slope of the progress curve. This procedure is only valid if the plot of product concentration as a function of time has been demonstrated to be linear within the time frame of the assay. However, this is not the only criterion that determines initial rate and steady-state kinetics. Linearity has been observed in many non-steady-state systems (Allison and Purich, 1979).

The general rule of thumb is that initial rate conditions prevail when the substrate concentration is still within 10% of its starting value. This value is clearly acceptable for reactions that are thermodynamically quite favorable and that do not exhibit any significant product inhibition. However, if the equilibrium constant for the reaction is not large, an estimate of the

degree of substrate-to-product conversion should be made. With smaller equilibrium constants, the initial-rate assay protocol necessarily must be more sensitive. In addition, if the product has very tight affinity for the enzyme, product accumulation can lead to significant errors in initial-rate determinations even in those cases where the degree of conversion is low. A preliminary test to assess this possibility is to look at initial rates in the absence and presence of a known amount of product. Where product inhibition is a problem, product can be depleted if an auxiliary enzyme system for doing this is present. Nevertheless, product inhibition can be an important regulatory scheme for an enzyme. Any kinetic characterization of an enzyme should include accurate measurements of the dissociation constants for all products. Product-inhibition studies can also assist the investigator in establishing mechanistic details of the protein undergoing evaluation (Rudolph, 1979).

In addition to establishing that the progress curve is truly linear, demonstrating that the degree of substrate depletion in the initial time course of the assay is low, and assessing the degree of product inhibition that may be present, the investigator should also demonstrate in the early stages of the kinetic studies that the rates observed are linear with respect to the total enzyme concentration ($[E]_{\text{total}}$). If true steady-state conditions are present, a plot of initial rates versus $[E]_{\text{total}}$ (in which the enzyme concentration is varied above and below the final value to be used in the initial-rate assays) should be linear.

Substrate Concentration Ranges

A wide range of substrate concentrations should be tested in preliminary studies. Such trials will provide an estimate of the Michaelis constant (K_m) and an assessment of whether substrate inhibition occurs at elevated substrate levels. These tests will also detect the possible existence of cooperativity. Once an estimate of the Michaelis constant has been made, a more detailed kinetic study can be made, in which the substrate concentration is typically varied from ~ 0.2 to 5.0 times the value of the apparent constant. It should be emphasized that reasonable estimates of the K_m value can only be made when initial rates have been obtained from substrate concentrations both below and above the true K_m value. For a one-substrate enzyme-catalyzed reaction:

$$K_m = [A](V_{\text{max}} - v)/v$$

Commonly Used
Techniques

A.3H.5

where $[A]$ is the substrate concentration, V_{\max} is the maximal velocity of the reaction, and v is the velocity of the reaction at the substrate concentration. A series of substrate concentrations with their respective initial rate velocities will provide an estimate of the Michaelis constant. In a standard double-reciprocal plot, the horizontal intercept is numerically equal to $-1/K_m$. For multisubstrate enzymes (see below), estimates of the Michaelis constants will be obtained from slope and intercept replots of the initial rate data. In addition, appropriate statistical treatment of the raw data should be provided (Wilkinson, 1961; Cleland, 1979a) for both K_m and V_{\max} values (or k_{cat} values, equal to $V_{\max}/[E]_{\text{total}}$, if those are reported). Note that, regardless of whether V_{\max} or k_{cat} values are reported, the total protein concentration, as well as the specific activity of the enzyme used, should be reported whenever possible.

With enzyme systems utilizing two substrates, the concentrations of both substrates must be varied as described above. Typically, three solutions are prepared. One solution contains one of the substrates at its highest level. A second solution contains the second substrate at its highest level. The third solution contains the buffer, all cofactors, metal ions, auxiliary

enzymes, and any other effectors, but no substrates. This third solution is used to prepare serial dilutions of the other two stock solutions. Thus, a matrix of initial rate measurements on both substrates can be evaluated from a single experimental trial. Figure A.3H.2 illustrates a typical double-reciprocal plot for a two-substrate enzyme-catalyzed reaction (where the two substrates are A and B). In this example, Michaelis constants for the two substrates can be obtained by constructing slope and/or intercept replots of the data. For example, plotting the slopes of the five lines as a function of the reciprocal of the concentration of B will yield a straight line with a horizontal intercept numerically equal to $-1/K_B$, where K_B is the Michaelis constant for B.

For enzymes that utilize three or more substrates, the protocol described above can become unwieldy. Two procedures have been described to address the design of kinetic studies for these proteins. In the first method (Frieden, 1959), one of the three substrates is held constant at a concentration above its respective Michaelis constant that is still subsaturating. The other two are then varied as described above. This same procedure is then followed with each of the remaining substrates. Thus, multisubstrate enzymes are reduced to pseudo-

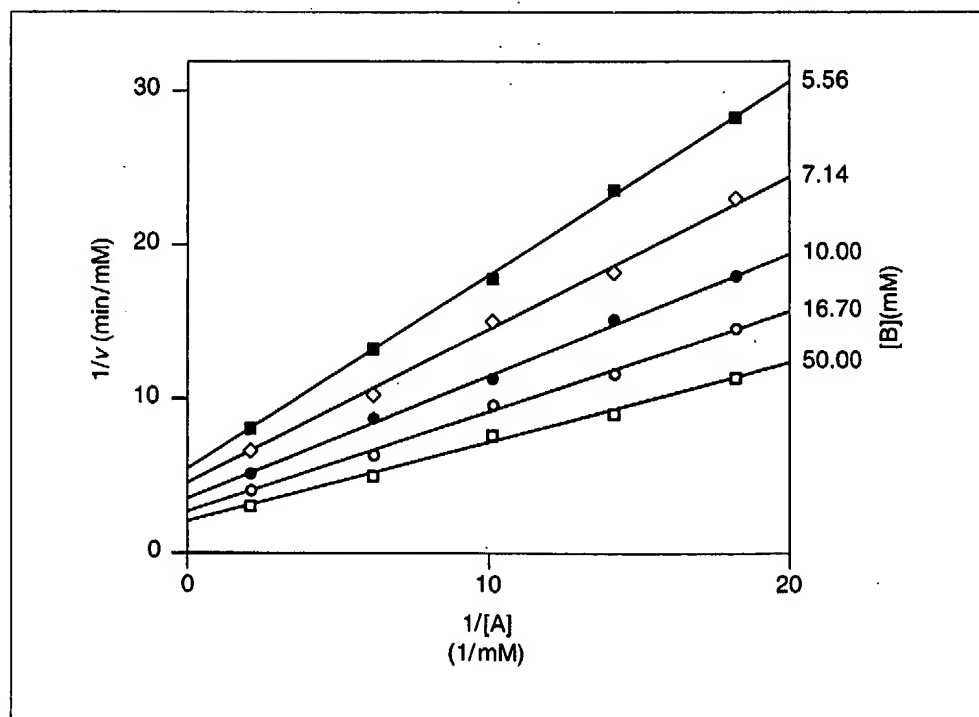


Figure A.3H.2 Double-reciprocal plot of a two-substrate enzyme-catalyzed reaction, where v = the velocity of the reaction and $[A]$ and $[B]$ represent the concentrations of substrates A and B respectively.

two-substrate systems. One distinct advantage of this procedure is that standard graphical presentations of the data thus produced (e.g., via double-reciprocal plots) are identical to that shown in Figure A.3H.2. However, the concentration value for the third substrate have to be provided. In the second procedure, the concentration of one substrate is varied while the other substrates are held at a constant ratio, although the absolute amounts are varied. This is then repeated for all remaining substrates until the concentration of each of the substrates has been varied (Fromm, 1967). One limitation with this approach is the difficulty in obtaining true Michaelis constants.

The presence of substrate inhibition can be an annoyance to an investigator who is attempting to determine the kinetic parameters of an enzyme as well as identify the binding pattern. However, the pattern of such inhibition can serve as a tool in understanding the kinetic and possibly the regulatory scheme for the enzyme. Substrate inhibition can occur as a result of a number of different causes—i.e., the formation of a dead-end complex with a particular enzyme form, the appearance of a different binding mechanism at elevated levels of substrate, the presence of an allosteric site for the substrate, or nonspecific inhibition that is due to an elevated ionic strength or another factor. When substrate inhibition occurs, in the case of multisubstrate enzymes, estimates of the K_i value (i.e., the dissociation constant for inhibition) for the substrate can be obtained from slope and intercept replots of the kinetic data. For enzymes having a single substrate, the data can be fit to an enzyme-rate expression containing a term for substrate inhibition ($v = V_{\max}[S]/\{K_m + [S] + [S]^2/K_i\}$). In addition, estimates of K_m , K_i , and V_{\max} can be obtained from double-reciprocal plots (Cleland, 1979b). Note that in such cases, double-reciprocal plots will be nonlinear, with the degree of curvature dependent on the magnitude of the K_i value. In these cases, it may be necessary to use a computer program to fit the data. Cleland (1979a) has provided a program to address substrate inhibition. In addition, one might consider utilizing a Marmasse plot (Marmasse, 1963). Although rarely used, this plot provides good estimates of K_m and K_i . A Marmasse plot is a plot of $1/v$ versus $(\alpha + 1/\alpha)$, where $\alpha = [A]/[A]_m$ and $[A]_m$ represents the concentration of substrate at which the velocity is highest (note that this is not V_{\max} , as substrate inhibition is present.) This plot, when combined with the observation that $[A]_m =$

$(K_m/K_i)^{1/2}$, will provide values for the kinetic parameters (Cleland, 1979b).

CONTINUOUS VERSUS STOP-TIME ASSAYS

As the best estimate for the initial rate of an enzyme-catalyzed reaction derives from the slope of the steady-state region of the progress curve, continuous assays are greatly preferable to single-point assays. If it is not possible to use a continuous assay, the investigator must demonstrate that the single-point assay is a true measure of the steady-state velocity of the reaction. In the single-point method, an aliquot of the reaction mixture is removed from the sample at a given time point after the reaction has been initiated (after a "blank" aliquot has been removed from the reaction mixture prior to the initiation step). Aliquots from at least four different time points are removed from the reaction solution; then the reaction is terminated and the amount of product formed (or substrate depleted) is determined. It should be ascertained that the termination procedure truly quenches the reaction. Once the steady-state relationship has been established, replicate kinetic analyses at each substrate concentration will provide sufficient data for a statistical analysis. The number of replicate samples that are assayed should also be reported.

COUPLED ENZYME ASSAYS

In many instances, continuous assays are made possible by the presence of auxiliary enzymes. Product formation is measured by having the product react further with another enzyme (or enzymes). Although such assay systems are convenient, precautions must be exercised to ensure that the initial rates observed reflect the enzyme that is undergoing investigation and not the auxiliary system. If the auxiliary system is influencing the initial-rate results, the reported values for the kinetic parameters will be in error and the double-reciprocals may exhibit considerable nonlinearity.

The product or products formed by the enzyme undergoing study clearly direct the choice of auxiliary enzyme(s). This coupling system should provide turnover in excess over that due to the enzyme being investigated. However, the degree of excess will vary depending upon the assay method, the nature of the coupling system, and the magnitude of the kinetic parameters for each auxiliary enzyme as measured under the same conditions being

observed with the target enzyme. Once this information is in hand, the amount of auxiliary enzyme needed can be calculated (McClure, 1969; Storer and Cornish-Bowden, 1974). Test runs should then be undertaken using both the calculated and higher levels of auxiliary enzymes to insure that identical rates are obtained, indicating that a sufficient excess has been achieved. The auxiliary enzyme system should also have minimal effect on cofactors and effectors required by the target enzyme.

Frequently, a small amount of product may be present in the reaction mixture as a contaminant. For example, commercial preparations of ATP often include a small amount of ADP. The contaminating product can be removed prior to initiation of the assay by incubating the reaction mixture with the auxiliary system. The system should also be checked to insure that no activity is observed in the absence of the auxiliary enzymes. Finally, care should be exercised when using commercial preparations for the auxiliary enzymes. Often these sources of proteins contain contaminating activities that may cause serious difficulties unless they are addressed.

BINDING STUDIES

Measurements of equilibrium ligand binding can frequently add to the characterization of an enzyme's binding mechanism, and also act as a tool in such procedures as molecular-receptor binding studies and radioimmunoassays. A wide variety of methods have been utilized in characterizing binding phenomena—e.g., gel filtration and equilibrium dialysis. The theory underlying this valuable tool can be found in any physical biochemistry text. Commonly, the binding data are analyzed with Scatchard plots (Scatchard, 1949) in which the number of moles of ligand bound per mole of total protein (v) are plotted versus the ratio of v to the free molar concentration of the ligand. These plots are frequently used to obtain values for the ligand dissociation constant, to identify interacting sites, and to estimate the binding capacity of the protein. Care should be exercised in this last use of the binding data, as it is easy to obtain a poor estimate of the stoichiometry of binding from Scatchard plots (Klotz, 1982, 1983). For example, a Scatchard plot of the binding of diazepam to benzodiazepine receptors is reported to provide an incorrect total number of receptor sites. Often, such problems are due to having only a few data points in the Scatchard plot at which the ligand concentration is above the half-maximum bind-

ing point. Just as in kinetic data, concentrations of the binding ligand both above and below this half-maximum point should be investigated (Klotz, 1982). Feldman (1983) has recently presented a statistical analysis associated with ligand binding data and Scatchard plots. As this paper points out, estimates of molar binding capacity should always be reported with the appropriate degree of statistical certainty. Without such statistical analyses, reports of the number of binding sites can be meaningless.

PRESENTATION OF INITIAL-RATE DATA

The description of the assay protocols used in connection with enzyme purifications, in clinical studies, and in all kinetic investigations should include a clear definition of the unit of enzyme activity. When possible, velocities should be expressed in terms of molarity changes with time, as molarity is an intensive variable. Other units, proportional to molarity, are in common use. In all cases, the conditions of the assay should be fully described, including the volume of the initiating aliquot, the specific activity, and the amount of enzyme used in each experiment. The international unit (U), defined as the conversion of 1 μ mol of substrate to product per minute, is in common use. Another unit of catalytic activity, the katal, corresponding to the transformation of one mole of substrate per second, is also in use. It has been suggested that the latter unit be restricted to catalytic activities measured in a clinical context (Dybkaer, 1979).

Initial-rate data is frequently best presented via graphical displays. These visual linearizations of kinetic information provide a straightforward evaluation of the binding sequence and mechanism, as well as a ready appraisal of the kinetic parameters, reversible inhibition, cooperativity (both allosteric and hysteretic), and many other steady-state parameters. Whenever possible, slope and/or intercept replots should also be provided in the graphical display, perhaps as insets of the initial rate data. There are three methods frequently employed for depicting kinetic data, with the double-reciprocal plot (i.e., $1/v$ versus $1/[A]$; see Fig. A.3H.2) being the most common. The other common plots are $[A]/v$ versus $[A]$ and v versus $v/[A]$. Wilkinson (1961) has discussed each of these plots in terms of statistics. When the data are unweighted, the three graphical presentations are not statistically equivalent. However, this problem is considerably mitigated by proper analysis using computer methods and data weighting

(Siano et al., 1975). As mentioned earlier, Cleland (1979a) has provided a number of computer programs for the analysis of data. Eisenthal and Cornish-Bowden (1974) have also introduced a novel method for graphical treatment of data that provides a useful tool for evaluating experimental error. An Eisenthal-Cornish-Bowden plot is based on the equation $(V_{\max}/v) - (K_m/[A]) = 1$. Instead of a particular set of data (i.e., a single initial rate velocity with its respective substrate concentration) being represented by a point in a Cartesian coordinate system, the velocity (v) is denoted along the vertical axis and the substrate concentration ($[A]$) is denoted along the horizontal axis. The two points thus plotted are then connected by a line. Thus, whereas in the other graphical procedures the velocity and substrate-concentration data are represented by points, in the Eisenthal-Cornish-Bowden plot the same data are represented by lines. These lines, obtained from a set of initial-rate experiments, will intersect about a point equivalent to $v = V_{\max}$ and $[A] = K_m$. Because median values of intersection points are used, outlier (i.e., aberrant) data will have minimal effects on estimates of the kinetic parameters. In addition, this plot is very useful in that calculations are not required and data do not have to be weighted. However, it does have limitations (Cornish-Bowden and Endrenyi, 1981, 1986). For example, the plot can become quite unwieldy with multisubstrate enzymes and in inhibition studies.

It has recently been suggested (Brand and Johnson, 1992; Johnson and Brand, 1994) that all of the graphical methods described above may be unnecessary now that personal computers and associated new methods of data analysis are available. For example, global analysis of biochemical data is a powerful tool that may prove to be quite useful in the analysis of enzyme kinetics (Beechem, 1992). However, these numerical methods have not yet been commonly applied to studies such as those of multisubstrate systems, equilibrium exchanges, or product inhibition. At this time, with proper statistical weighting of the data, multisubstrate systems are more readily appraised by one of the common graphical procedures. More importantly, these visual depictions of initial-rate data are a clear aid to any individual reviewing the kinetic experiments.

CONCLUDING REMARKS

Initial-rate assays are extremely useful tools in the detailed characterization of an enzyme. The behavior, mechanism, and regulatory prop-

erties of an enzyme can be readily assessed by straightforward assays. Such assays can frequently be completed within a day, once preliminary runs have identified the magnitude of the kinetic parameters and revealed those factors that affect activity. However, the details of the kinetic mechanism and regulatory properties of the enzyme can easily be obscured by poor assay design. The structural/functional role of an enzyme, as well as its regulation and control, can only be assessed by a reliable initial-rate assay. In addition, the data assembled from these rigorous studies permit the development of more reliable and sensitive assay protocols for protein purification and identification.

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Describes a number of isotopic probes for studying enzyme mechanisms and contains valuable chapters on allosterism, hysteresis, immobilized systems, and processivity.

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Describes methods for characterizing intermediates in enzyme-catalyzed reactions and using stereochemical probes for enzyme mechanisms; includes additional initial-rate and inhibitor methods and discusses further uses of isotopic probes.

Purich, D.L. (ed.) 1995. Enzyme kinetics and mechanism, part D. *Methods in Enzymol.* 249:3-662.

Covers in detail specialized topics in enzyme kinetics including transition-state approaches, kinetic probes with site-directed mutagenesis, partition analysis, positional isotope exchange, interfacial catalysis, and hydrogen tunneling.

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Constructing Recombinant DNA Molecules by the Polymerase Chain Reaction

UNIT 3.17

Any two segments of DNA can be ligated together into a new recombinant molecule using the polymerase chain reaction (PCR). The DNA can be joined in any configuration, with any desired junction-point reading frame or restriction site, by incorporating extra nonhomologous nucleotides within the PCR primers. Cloning by PCR is often more rapid and versatile than cloning with standard techniques that rely on the availability of naturally occurring restriction sites and require microgram quantities of DNA. It is not necessary to know the nucleotide sequence of the DNA being subcloned by this technique, other than the two short flanking regions (~20 bp) that serve as anchors for the two oligonucleotide primers used in the amplification process. Moreover, PCR can be performed on low-abundance or even degraded DNA (or RNA) sources.

This unit describes using PCR to construct hybrid DNA molecules. The main objective is to give an overview of how PCR can be exploited to accomplish numerous cloning strategies; it is assumed that the reader is already familiar with basic molecular biology techniques including PCR amplification (UNIT 15.1) and subcloning (UNIT 3.16). The basic protocol outlines the PCR amplification and cloning strategies. A troubleshooting guide for problems most frequently encountered in PCR cloning, and three specific examples of this technique—for creating (1) in-frame fusion proteins, (2) recombinant DNA products, and (3) deletions and inversions by inverse PCR—are presented in the Commentary.

SUBCLONING DNA FRAGMENTS

In this protocol, synthetic oligonucleotides incorporating new unique restriction sites are used to amplify a region of DNA to be subcloned into a vector containing compatible restriction sites. The amplified DNA fragment is purified, subjected to enzymatic digestion at the new restriction sites, and then ligated into the vector. Individual subclones are analyzed by restriction endonuclease digestion and either sequenced or tested in a functional assay. The procedure is summarized in Figure 3.17.1.

Materials

Template DNA (1 to 10 ng of plasmid or phage DNA; 20 to 300 ng of genomic or cDNA)

Oligonucleotide primers (0.6 to 1.0 mM; UNIT 8.5)

Mineral oil

TE-buffered phenol (UNIT 2.1) and chloroform

100% ethanol

TE buffer, pH 8.0 (APPENDIX 2)

Klenow fragment of *E. coli* DNA polymerase I (UNIT 3.5)

Vector DNA

Calf intestinal phosphatase (UNIT 3.10)

Additional reagents and equipment for phosphorylating synthetic oligonucleotides (UNIT 3.10), enzymatic amplification of DNA by PCR (UNIT 15.1), agarose and polyacrylamide gel electrophoresis (UNITS 2.5 & 2.7), DNA extraction and precipitation (UNIT 2.1), purification of DNA by glass beads, electroelution from agarose gels, or from low-gelling/melting temperature agarose gels (UNIT 2.6), restriction endonuclease digestion (UNIT 3.1), ligation of DNA fragments (UNIT 3.16), transformation of *E. coli* (UNIT 1.8), plasmid DNA minipreps (UNIT 1.6), and DNA sequence analysis (UNIT 7.4)

BASIC PROTOCOL

Enzymatic
Manipulation
of DNA and RNA

3.17.1

Contributed by Elaine A. Elion

Current Protocols in Molecular Biology (1993) 3.17.1-3.17.10

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Supplement 24

Amplify the target DNA

1. Prepare the template DNA. If using an impure DNA preparation (i.e., not purified by CsCl gradients), heat sample 10 min at 100°C to inactivate nucleases.

Plasmid, phage, genomic, or cDNA, obtained from either rapid preparations or purified on CsCl gradients, can be used as the source of target DNA.

2. Prepare the oligonucleotide primers. If the PCR product is to be cloned by blunt-end ligation, phosphorylate the 5' hydroxyl of the oligonucleotide primers.

A 5' phosphate on the ends of the PCR products will be needed to form the phosphoester linkage to the 3'OH of the vector during ligation. This step is essential if the vector has been treated with a phosphatase.

Because the purity of the oligonucleotides does not seem to affect the PCR reaction, primer purification (as detailed in UNIT 8.5) may not be necessary.

3. Set up a standard amplification reaction and overlay with mineral oil as described in UNIT 15.1. Carry out PCR in an automated thermal cycler for 20 to 25 cycles under the following conditions: denature 60 sec at 94°C, hybridize 1 min at 50°C, and extend 3 min at 72°C. Extend an additional 10 min at 72°C in the last cycle to make products as complete as possible.

Include negative controls of no template DNA and each oligonucleotide alone, as well as several different oligonucleotide:template ratios.

For a discussion of optimization of amplification conditions see UNIT 15.1. A thermostable DNA polymerase with 3'→5' exonuclease proofreading activity can be used instead of Taq DNA polymerase to reduce the amount of nucleotide misincorporation during amplification. Pfu DNA polymerase (Stratagene) and Vent DNA polymerase (New England Biolabs) have this activity (follow manufacturers' instructions).

Recover the amplified fragment

4. Analyze an aliquot (e.g., 4 to 8 µl) of each reaction mix by agarose or polyacrylamide gel electrophoresis to verify that the amplification has yielded the expected product.
5. Recover amplified DNA from PCR reaction mix. Remove mineral oil overlay from each sample, then extract sample once with buffered chloroform to remove residual mineral oil. Extract once with buffered phenol and then precipitate DNA with 100% ethanol.

Carrier tRNA may be added during precipitation if desired.

6. Microcentrifuge DNA 10 min at high speed, 4°C. Dissolve pellet in 20 µl TE buffer. Purify desired PCR product from unincorporated nucleotides, oligonucleotide primers, unwanted PCR products, and template DNA using glass beads, electroelution, or phenol extraction of low gelling/melting temperature agarose.

Unused oligonucleotide primers can inhibit the ability of the restriction enzymes to digest the amplified PCR product. Amplified DNA that is greater than 100,000 Da (>150 bp) can be rapidly separated from the primers using a Centricon 100 microconcentrator unit from Amicon (follow manufacturers' instructions). If the gel analysis in step 4 shows that amplification yielded only the desired PCR product, this microconcentrated DNA can be used directly for cloning.

Prepare amplified fragment and vector for ligation

- 7a. If the PCR fragment is to be cloned by blunt-end ligation, repair the 3' ends with DNA polymerase I (Klenow fragment).

This step is necessary because Taq DNA polymerase adds a nontemplated nucleotide (usually dA) to the 3' ends of PCR fragments.

- 7b. If primers contain unique restriction sites, digest half the amplified DNA in 20 μ l with the appropriate restriction enzyme(s). Use an excess of enzyme, and digest for several hours.

Reserve the undigested half for future use, if necessary.

8. Prepare the recipient vector for cloning by digesting 0.2 to 2 μ g in 20 μ l with compatible restriction enzymes. If necessary, treat vector DNA with calf intestinal phosphatase (UNIT 3.1) to prevent recircularization during ligation.
9. Separate the linearized vector from uncut vector by agarose or low-gelling/melting temperature gel electrophoresis. Recover linearized vector from the gel by adsorption to glass beads, electroelution, or phenol extraction of low-gelling/melting temperature agarose.

Ligate amplified fragment and vector

10. Ligate the PCR fragment into the digested vector following the procedure outlined in UNIT 3.16.
11. Transform an aliquot of each ligation into *E. coli*. Prepare plasmid miniprep DNA from a subset of transformants.

Analyze recombinant plasmids

12. Digest the plasmid DNA of the selected transformants with the appropriate restriction endonuclease. Analyze the digestions by agarose gel electrophoresis to confirm fragment incorporation.
13. Sequence the amplified fragment portion of the plasmid DNA to check for mutations. Alternatively, screen the subset of transformants using a biochemical or genetic functional assay if available.

This analysis is critical because the Taq DNA polymerase can introduce mutations into the amplified fragment.

COMMENTARY

Background Information

The main benefit of cloning by PCR is that unique restriction sites can be introduced on either side of any segment region of amplified DNA to allow its ligation into a recipient vector (Mullis and Faloona, 1987; Chapter 15) in any configuration. The incorporation of additional nucleotides at the 5' ends of the oligonucleotide primers permits the creation of novel restriction sites or changes in reading frame and coding sequence. The oligonucleotide primers can also be designed to contain mismatches, deletions, or insertions in the region of homology (UNIT 8.5). However, it is not necessary to always incorporate a new restriction site in the primer. Amplified PCR fragments can also be subcloned by blunt-end or sticky-end ligation using preexisting restriction sites within the amplified DNA. For example, PCR might be used to amplify a target DNA that already contains appropriate restriction sites, but is available in limited quantities. Finally, sequential polym-

erase chain reactions can be used to generate more complex recombinant PCR products which can subsequently be subcloned into a recipient vector.

The most obvious disadvantage of PCR cloning is the need to verify that the subcloned PCR product does not contain mutations generated during the polymerase chain reaction. In cases where longer DNA segments (i.e., >1 kb) are being amplified, it may be more advantageous to use a polymerase which has a 3'→5' exonuclease activity (e.g., Pfu polymerase, Stratagene) to reduce the chances of generating mutations. After subcloning, several independent PCR products should be analyzed by DNA sequencing to be sure that the recombinant DNA molecule is not mutated. Sequencing can be laborious when a large fragment of DNA is subcloned. However, subcloned PCR products can be prescreened by either biological or biochemical functional assays if they are available. In some instances, it may be more desir-

able to break down the cloning into several steps that might involve the introduction of a needed restriction site within a short piece of DNA first.

Critical Parameters

In general, the DNA preparation, purification, and ligation guidelines outlined in UNIT 3.16 should be applied to PCR cloning to ensure recovery of the desired ligation products. However, the following points deserve special consideration.

Design of oligonucleotide primers. Primers should only hybridize to the sequence of interest. This can be predicted in instances where sequence information is available. In general, primers with homology of 16 to 20 nucleotides to the target DNA and a GC content of ~50% should be chosen. A longer oligonucleotide of ~25 nucleotides should be used for AT-rich regions. In instances where genomic DNA is the source of the target DNA, the oligonucleotide primers should contain at least 20 nucleotides of homology to the target DNA to ensure that they anneal specifically (Arnheim and Erlich, 1992).

When using primers to introduce a specific restriction site, a sequence within the target DNA should be selected that requires the addition of the fewest noncomplementary nucleotides to create the new site, if possible. Special consideration should be given to the choice of site itself, as restriction endonucleases vary in their ability to cleave recognition sequences within ten nucleotides of the end of a DNA duplex (consult Table 8.5.1 for the efficacies of different restriction enzymes in cleaving terminal recognition sequences). It is also recommended that four to five additional nucleotides be added on the 5' side of the restriction site in the primer. Because DNA duplexes "breathe" at termini, potentially interfering with the ability of a restriction enzyme to cleave (Innis et al., 1990), it is useful to use the GCGC "clamp" sequence that is most thermostable (Sheffield et al., 1989).

Finally, the sequence of the primer should be checked for internal complementarity to avoid secondary structure formation that will interfere with hybridization of the primer to the target DNA. The 3' ends of the two primers being used must not be complementary, so that the formation of primer-dimers that will compete with the synthesis of the desired PCR product will be avoided.

Additional details on primer design are discussed in UNIT 15.1.

DNA polymerase. Commercially available *Taq* DNA polymerase (Perkin-Elmer Cetus) lacks the 3'→5' proofreading exonuclease activity used by DNA polymerase I Klenow fragment and T4 DNA polymerase to reduce error frequency (Kornberg, 1992). This absence of proofreading activity in *Taq* DNA polymerase is thought to result in a heightened error frequency. Old estimates indicate that the average rate of misincorporation is 8.5×10^{-6} nucleotides per cycle (Goodenow et al., 1989; Fucharoen et al., 1989). Two other thermostable DNA polymerases possessing proofreading 3'→5' exonuclease activity have recently become commercially available: Pfu DNA polymerase, purified from *Pyrococcus furiosus* (Stratagene) and Vent DNA polymerase, purified from *Thermococcus litoralis* (New England Biolabs and Promega). Both are more thermostable than *Taq* DNA polymerase. Pfu DNA polymerase is 12-fold more accurate than *Taq* DNA polymerase, as assayed by the method of Kohler et al. (1991). Vent DNA polymerase is 4-fold more accurate than *Taq* DNA polymerase (Cariello et al., 1991). Although it is difficult to compare the relative error frequencies of three enzymes because they were assayed by different methods, the use of either Vent or Pfu DNA polymerases may reduce the amount of misincorporation.

Removal of unincorporated nucleotide triphosphates. It is recommended that the amplified PCR fragment be purified from unincorporated nucleotides and primers. Any method of purification that involves electrophoresis can also separate the desired PCR product from any undesired DNA species produced during amplification. Typical methods of DNA purification include electrophoresis through low-gelling/melting temperature agarose or electrophoresis through agarose followed by DNA purification by electroelution or adsorption to glass beads (UNIT 2.6). However, amplified DNA can be more rapidly purified from unincorporated nucleotide triphosphates and primers using a Centricon microconcentration unit (Amicon). The disadvantage of using the microconcentrator is that undesirable PCR products and the starting template DNA will copurify with the amplified PCR fragment.

Troubleshooting

PCR amplification. The use of appropriately designed primers should allow the amplification of the DNA segment of interest. Occasionally, however, primers may not be specific, leading to the amplification of undesired DNA

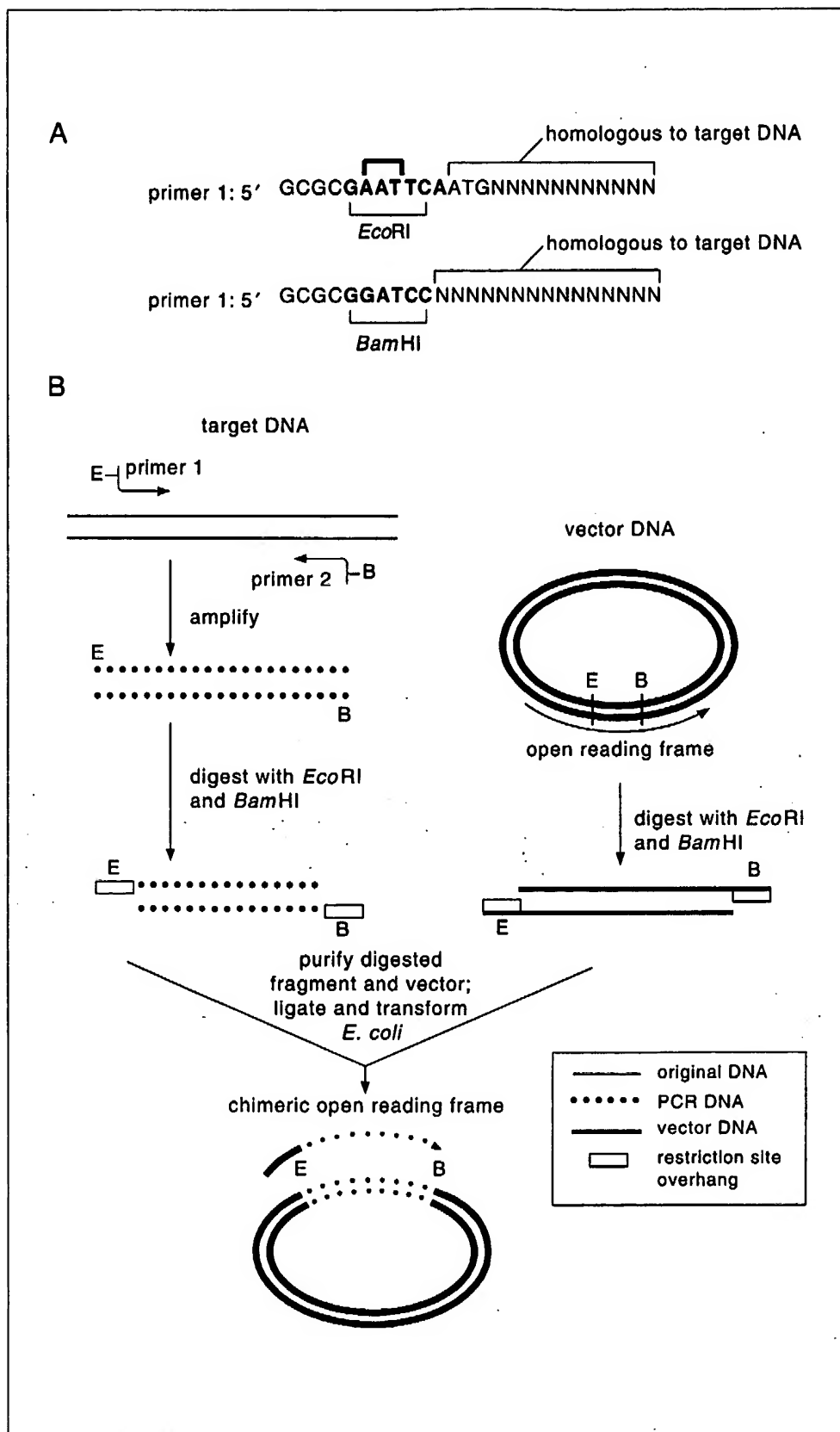


Figure 3.17.1 Introducing unique restriction sites and creating an in-frame fusion protein by PCR. Abbreviations: E, *EcoRI*; B, *BamHI*. For a full description, see Example 3.17.1 in Commentary.

segments. The specificity of primer to template hybridization will depend upon temperature and salt (see *UNITS 15.1 & 6.4* for a thorough discussion). The highest annealing temperature possible should be used to reduce nonspecific associations. Some nonspecific amplifications can be avoided by employing a "hot-start" technique—i.e., adding the DNA polymerase to a prewarmed sample (D'Aquila et al., 1991). In addition, purifying the PCR product by gel electrophoresis will help ensure that the proper DNA fragment is subcloned. If the synthesized primer does not bind with specificity, it may be simplest to have another one synthesized.

In setting up the amplification cycle, keep in mind that for cloning, fidelity is more important than yield, so it is better to keep a low cycle number and not to raise the MgCl_2 concentration too much. 1.5 mM MgCl_2 in the amplification buffer should be sufficient for most primers.

Cloning of the amplified fragment. "Sticky-end" ligation of the amplified DNA can sometimes be difficult, due to poor cutting of the terminal restriction site by the desired restriction endonuclease. Careful choice of restriction sites and the addition of extra nucleotides to the 5' end of the primer (see critical parameters) will facilitate digestion.

Other common explanations for poor cutting by restriction endonucleases include:

- (1) Blockage of the duplex terminus by bound *Taq* DNA polymerase. If this is the case, the amplified DNA can be treated with proteinase K to remove associated protein. This is done by adding 50 $\mu\text{g}/\text{ml}$ proteinase K in 10 mM Tris-Cl (pH 7.8)/5 mM EDTA/0.5% (v/v) SDS to the sample and incubating 30 min at 37°C. The sample must be extracted with phenol/chloroform (*UNIT 2.1*) to remove the proteinase K.

- (2) Inefficient extension by *Taq* DNA polymerase. The nonduplex ends generated in this fashion can be repaired by filling in with Klenow fragment (*UNIT 3.5*).

- (3) An insufficient number of extra nucleotides 5' to the restriction site in the primer. In this case, the primer should be resynthesized. Prolonged (typically overnight) DNA digestion should also be tried.

- (4) 5' terminal breathing of duplex DNA. Duplex formation can be stabilized by the inclusion of 0.1 mM spermidine in the digestion reaction.

An alternative approach is to internalize the restriction site by concatemering the PCR products prior to restriction endonuclease di-

gestion (Jung et al., 1990). To do this, 5' phosphorylated oligonucleotide primers are used in the PCR amplification, or following amplification, the DNA fragment is phosphorylated using T4 polynucleotide kinase (*UNIT 3.10*). The amplified fragments are then concatemered by ligation using T4 DNA ligase (*UNIT 3.16*) prior to restriction endonuclease treatment. Aliquots of the amplified DNA before and after concatemering, and after digestion, can be compared on an agarose gel to confirm that the procedure worked.

Blunt-end ligations are often inhibited by nonflushed ends in the PCR fragment, due to the presence of a nontemplate-directed nucleotide (usually dATP) added by *Taq* DNA polymerase (Clark, 1988). Treatment of the PCR fragment with Klenow polymerase in the presence of dNTPs (*UNIT 3.5*) to make the ends flush should circumvent this problem. In the event that this simple approach does not work, the PCR fragment can be subcloned into a *Mst*II (or *Bsu*36 I) site (CC↓TNAGG) which leaves a 5' dT overhang. This approach requires a recipient vector with a unique *Mst*II site.

Anticipated Results

All of the cloning approaches outlined are reliable and should result in efficient recovery of the desired recombinant molecules.

Time Considerations

Once the oligonucleotides have been synthesized, the PCR amplification, purification, ligation, and transformation steps can all be done within 2 days. The appropriate subclones can then be sequenced or tested in a functional assay immediately thereafter.

EXAMPLES

Example 3.17.1: Creating In-Frame Fusion Proteins by PCR

PCR cloning is particularly useful for creating in-frame fusions between two open reading frames, as is often done for synthesizing fusion proteins with *E. coli* expression vectors (Chapter 16). The essence of this type of subcloning involves incorporating additional noncomplementary nucleotides within the oligonucleotide primer that will encode the junction sequences of the amplified PCR fragment. Consider the introduction of a unique *Eco*RI site into a piece of target DNA that is to be fused with an open reading frame in the recipient vector, as depicted in Figure 3.17.1.

For this experiment, the primers should be

designed as indicated in panel A. Each primer is designed to contain a unique restriction site not present within the target DNA. The primer carrying the *Eco*RI site contains an additional nucleotide (shown in bold) to allow the ATG of the amplified target DNA to be in-frame with the open reading frame in the vector (bold bracket). The second primer contains a unique *Bam*HI site. Both oligonucleotide primers are designed to be homologous to and anneal with ~20 nucleotides of DNA flanking the target DNA. They are oriented such that their 3' hydroxyl ends point toward the target DNA. These unique restriction sites are 5' to the region of the primer that is homologous to the target DNA. Each new restriction site is separated from the 5' end of the oligonucleotide by four additional nucleotides to facilitate enzymatic digestion of the amplified DNA. Shown in this example is a GC clamp (Myers et al., 1985) which favors duplex formation at the ends of the amplified fragment.

Panel B depicts the sequence of events in this experiment. Following primer annealing and PCR amplification, the amplified DNA is first digested with restriction endonucleases that cleave at the new restriction sites, then purified by gel electrophoresis. The recipient vector DNA is digested with either the same or compatible restriction endonucleases and is purified before being ligated to the recipient vector.

In this example, one oligonucleotide contains additional bases to create an in-frame fusion with the plasmid-borne open reading frame. More elaborate primers can be designed to include additional restriction sites in different reading frames to allow subcloning of the same PCR fragment into multiple recipient fusion vectors that may have nonidentical cloning sites. This is efficient both in terms of labor and the cost of having to synthesize a new oligonucleotide primer. Note that an ATG can also be incorporated into the 5' oligonucleotide primer to create an open reading frame with a new translational start (e.g., in the construction of a promoter-exon fusion).

Example 3.17.2: Creating a Recombinant DNA Molecule by Sequential PCR Amplifications

Consider creating a chimeric DNA molecule by sequential polymerase chain reactions rather than by ligation. This technique is useful for complex cloning schemes that involve fusing together more than two pieces of DNA, as depicted in the creation of the gene fusion

shown in Figure 3.17.2. In this example, two PCR products are made from noncontiguous regions of DNA (that are also nonhomologous) in separate reactions.

Two of the first-round amplification primers are designed to contain 5' extensions that are homologous to a portion of the other target gene (see primers 1b and 1c). In this example, the primers for target gene 1 are labeled as 1a and 1b. Primer 1a contains a unique *Eco*RI site; primer 1b contains a 5' extension that is homologous to a region in target gene 2 that will be amplified (thin line of arrow). Primers 1c and 1d are for amplifying target gene 2: primer 1c contains a 5' extension that is homologous to a portion of target gene 1 that will be amplified (bold line of arrow), and primer 1d contains a unique *Bam*HI restriction site.

Because primers 1b and 1c contain complementary 5' extensions, two PCR products containing a region of overlapping homology are generated. The two PCR fragments are purified away from the primers, then mixed together and annealed by denaturation and renaturation. Four DNA species are generated in this reaction: two heteroduplexes associating at the region of overlapping homology and two parental homoduplexes. The recessed 3' ends of the heteroduplexes are extended by *Taq* DNA polymerase to produce a single fragment that is equal in length to the sum of the two overlapping fragments.

In a second round of amplification, the combined heteroduplex DNA species is amplified by adding the outside set of primers (1b and 1d) to the PCR assay. These primers will now have complete homology to the amplified heteroduplex DNA species. (Note that the parental homoduplexes will not be amplified because only one of each outside primers will anneal to each parental homoduplex.)

The complementary primers used in the first polymerase chain reaction step can be designed to either insert a restriction site at the junction between the joined PCR products, or alter a reading frame.

Example 3.17.3: Inserting a Restriction Site by Inverse PCR

Consider deleting a segment of DNA from a plasmid by inserting a unique restriction site by inverse PCR as depicted in Figure 3.17.3. Divergent primers containing the same novel restriction site (R) are annealed to two portions of the plasmid. (Note that R can be any restriction site not found in the plasmid.) The primers are oriented with their 3' ends facing away from

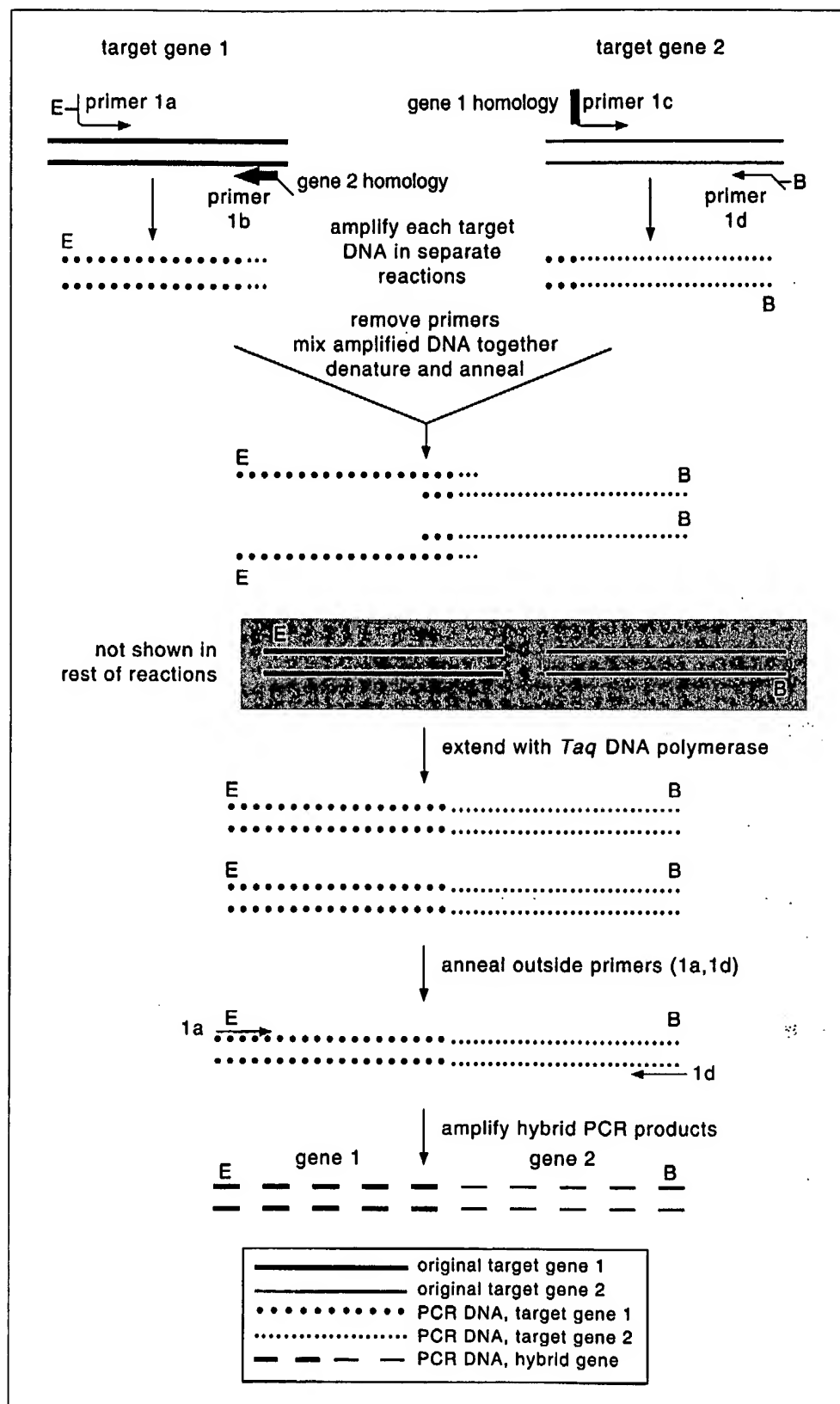


Figure 3.17.2 Creating a recombinant DNA molecule by sequential PCR amplifications. Primer 1b has a region of homology to target gene 2 (open box); primer 1c has a region of homology to target gene 1 (closed box). Abbreviations: E, *Eco*RI; B, *Bam*HI. For a complete description, see Example 3.17.2 in Commentary.

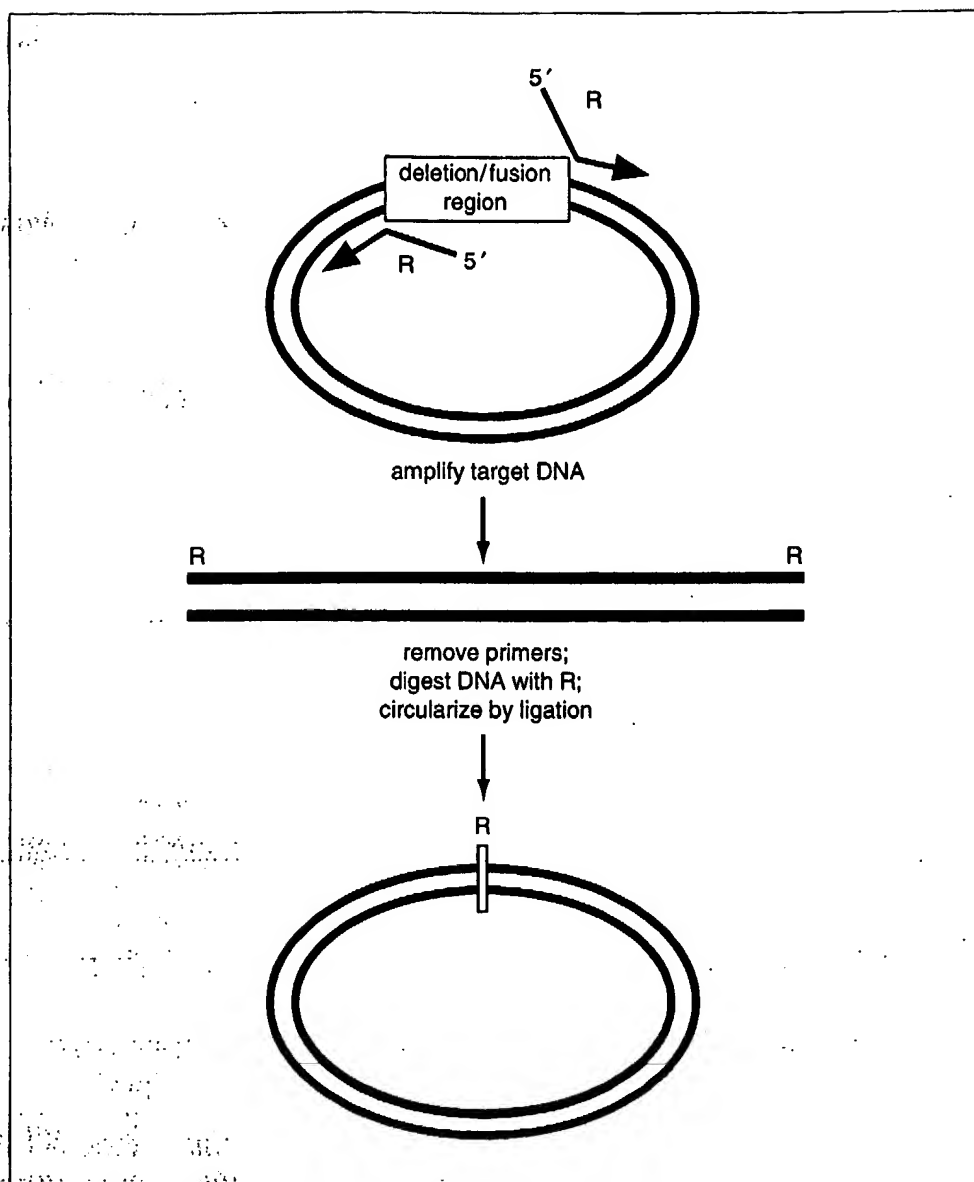


Figure 3.17.3 Inserting a restriction site by inverse PCR. Abbreviation: R, restriction enzyme not found in target plasmid. For a complete description, see Example 3.17.3 in Commentary.

each other so that sequences flanking the region to be deleted will be amplified. The full-length PCR product is purified and digested at the new restriction site and the ends are ligated in a unimolecular reaction. The amount of spacing between the two primers will determine whether the final product contains all of the original sequence or a deletion. Insertions can also be made at specific sites by including 5' extensions in the primers. Mismatches within the body of the primer can also be used to introduce mutations.

This method is useful for rapid introduction of desired restriction sites, and is limited only by the size of the plasmid and the ability of

DNA polymerase to synthesize complete products. This methodology allows the amplification of DNA flanking a region of known sequence. It is also useful for cloning DNA that has not yet been sequenced and for making hybridization probes (Ochman et al., 1990).

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Key Reference

Innis et al., 1990. See above.

Provides an in-depth analysis of PCR methods and techniques.

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CHAPTER 6

Screening of Recombinant DNA Libraries

INTRODUCTION

The usual approach to isolating a recombinant DNA clone encoding a particular gene or mRNA sequence is to screen a recombinant DNA library. As described in Chapter 5, a recombinant DNA library consists of a large number of recombinant DNA clones, each one of which contains a different segment of foreign DNA. Since only a few of the thousands of clones in the library encode the desired nucleic acid sequence, the investigator must devise a procedure for identifying the desired clones. The optimal procedure for isolating the desired clone involves a positive selection for a particular nucleic acid sequence. If the desired gene confers a phenotype that can be selected in bacteria, then the desired clone can be isolated under selective conditions (UNIT 1.4). However, most eukaryotic genes and even many bacterial sequences do not encode a gene with a selectable function. Clones encoding nonselectable sequences are identified by screening libraries: the desired clone is identified either because (1) it hybridizes to a nucleic acid probe, (2) it expresses a segment of protein that can be recognized by an antibody, or (3) it promotes amplification of a sequence defined by a particular set of primers.

Screening libraries involves the development of a rapid assay to determine whether a particular clone contains the desired nucleic acid sequence. This assay is used first to identify the recombinant DNA clone in the library and then to purify the clone (see Fig. 6.0.1). Normally, this screening procedure is performed on bacterial colonies containing plasmids or cosmids or on bacteriophage plaques. To test a large number of clones at one time, the library is spread out on agarose plates (UNIT 6.1), then the clones are transferred to filter membranes (UNIT 6.2). The clones can be simultaneously hybridized to a particular probe (UNITS 6.3 & 6.4) or bound to an antibody (UNITS 6.7 & 6.11). When the desired clone is

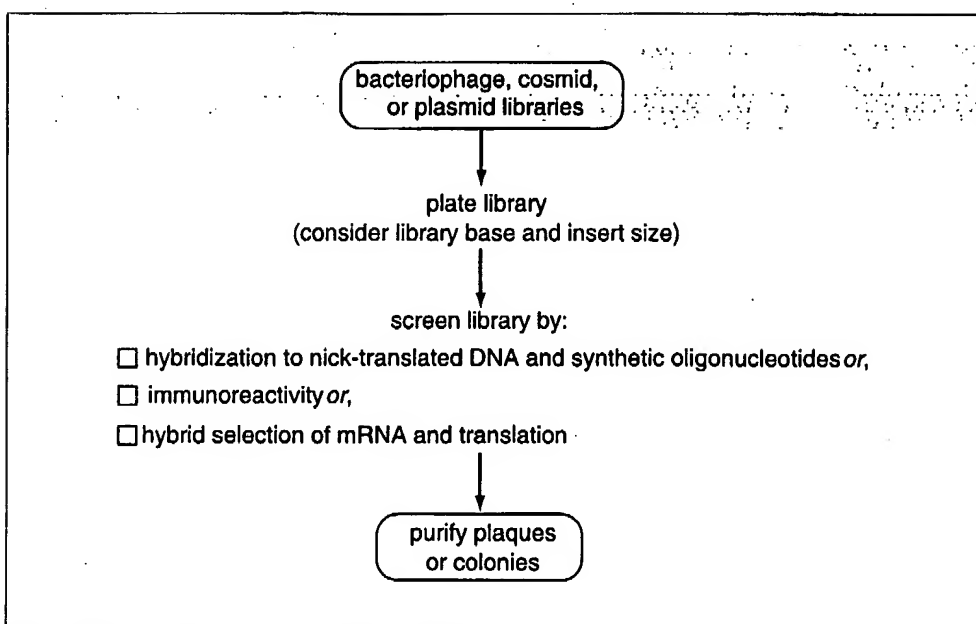


Figure 6.0.1 Flow chart for screening libraries.

first identified, it is usually found among many undesirable clones; an important feature of library screening is the isolation of the desired clones (UNITS 6.5, 6.6 & 6.12). Another method for identifying the desired clone involves hybrid selection (UNIT 6.8), a procedure by which the clone is used to select its mRNA. This mRNA is characterized by its translation into the desired protein. Libraries consisting of large genomic DNA fragments (~1 Mb) carried in yeast artificial chromosome (YAC) vectors have proven to be tremendously useful for genome analysis. In general, these libraries (which are usually produced by large "core" laboratories) are initially screened using a locus-specific PCR assay (UNIT 6.9); the clone resulting from the initial round of screening is subsequently analyzed by more conventional hybridization methods (UNIT 6.10).

To screen a DNA library, one must first devise the screening procedure. The next important choice is the selection of a recombinant DNA library. When choosing which library to screen the investigator should consider whether he or she wants to isolate clones encoding the gene or the mRNA sequence. cDNA clones encode the mRNA sequence and allow prediction of the amino acid sequence, whereas genomic clones may contain regulatory as well as coding (exon) and noncoding (intron) sequences. The differences between genomic and cDNA libraries are discussed in Chapter 5.

Another critical parameter to be determined before proceeding with a library screen is the number of clones in the library that must be screened in order to identify the desired clone. That is, what is the frequency of the desired clone in the library? This frequency is predicted differently for genomic and cDNA libraries, as described below.

Screening a genomic library. In general, genomic libraries can be made from DNA derived from any tissue, because only two copies of the gene are present per cell or per diploid genome. The predicted frequency of any particular sequence should be identical to the predicted frequency for any other sequence in the same genome. The formula for predicting the number of clones that must be screened to have a given probability of success is presented in UNIT 5.1. This number is a function of the complexity of the genome and the average size of the inserts in the library clones. For amplified libraries, the base (see UNIT 5.1) must exceed this number. Usually about 1 million bacteriophage clones or 500,000 cosmid clones must be screened to identify a genomic clone from a mammalian DNA library. Many of the clones that are screened from an amplified library will be screened more than once; the total number of clones that must be screened is 30 to 40% greater than the number calculated by the formula.

Screening a cDNA library. The optimal cDNA library is one made from a particular tissue or cell that expresses the desired mRNA sequence at high levels. In highly differentiated cells, a particular mRNA may comprise as many as 1 of 20 of the poly(A)⁺ mRNA molecules, while some mRNAs are either not present at all or comprise as low as 1 molecule in 100,000 poly(A)⁺ mRNA molecules. When choosing a cDNA library the investigator must make every effort to obtain a library from a cell where the mRNA is being expressed in large amounts. Of course, the number of clones that must be screened is determined by the abundance of the mRNA in the cell. The amount of protein that is found in the cell is frequently a good indicator of the abundance of the mRNA. Thus, proteins that comprise 1% of the total cell protein are made by mRNAs that usually comprise 1% of the total poly(A)⁺ mRNA, and the desired cDNA clones should comprise about 1% of the clones in the cDNA library.

Screening a YAC library. In the typical genomic libraries maintained in *E. coli* (described in Chapter 5), the size of the insert is limited to 20 to 25 kb for lambda vectors or to 40 to 45 kb for cosmid vectors. Yeast artificial chromosome (YAC) vectors, by contrast, are designed to carry much larger genomic DNA fragments and thereby facilitate genomic analysis, with inserts ranging from 0.3 to ~1 Mb in size. Conventional screening of YAC

libraries by hybridization is difficult, both because of the unfavorable signal-to-noise ratio and the sheer numbers of replica films required to represent an entire library.

For example, a standard YAC library representing 5 to 8 genome equivalents requires over 500 microtiter plates (and corresponding filters for screening by hybridization). Thus, most core laboratories screen YAC libraries using a locus-specific PCR assay whose primers define a particular sequence. The PCR screening is initially performed using pools (representing up to 4 microtiter plates or 384 YAC clones) or superpools (representing up to 20 microtiter plates or nearly 2000 clones), followed by subsequent rounds of screening to narrow down the possible candidates.

Specialized screening strategies. For particular applications, there exist specialized approaches to screening. For example, cloned cDNAs encoding cell surface or intracellular proteins can be identified by expression screening, involving rounds of transient expression of a library and subsequent screening by immunoselection (UNIT 6.11). The technique of recombination-based screening provides a rapid and efficient approach for screening a complex genomic library in bacteriophage lambda (UNIT 6.12). The library is screened for homology against a plasmid carrying a particular cloned target sequence. If homology exists, a recombination event occurs, resulting in integration of the plasmid into the phage, and the recombinant is isolated by genetic selection.

General considerations. When selecting the library it is critical that the base be larger than the number of clones to be screened. One problem with predicting the number of clones to screen is that most libraries are amplified and in the process of amplifying the library some clones are lost while others may grow more rapidly. Thus, if the desired clone is not found in a particular library, another independent library should be screened.

Having selected the library, the investigator is ready to begin screening for the desired clone. The technologies used to screen libraries are mostly extensions of the techniques that have been described earlier in the manual. Libraries are plated out, transferred to nitrocellulose filters, and hybridized to ³²P-labeled probes or bound to antibodies. The major problem associated with this technique is that "false" positives can be identified: the probe may hybridize to clones that do not encode the desired sequence. Approaches to minimize this problem are discussed in UNIT 6.7. A second source of undesired clones arises from the power of the screening procedures that are normally used to screen these libraries. The investigator will be screening as many as one million clones. If the library contains any contaminating recombinant DNA clones that have been previously grown in the laboratory, it will be identified in the screening procedure. Thus, extreme care must be exercised to prevent contamination of the library with previously isolated recombinant clones. Despite these problems the ability to screen large DNA libraries to isolate the desired clone provides a powerful tool for molecular biologists.

J.G. Seidman

PLATING LIBRARIES AND TRANSFER TO FILTER MEMBRANES

SECTION I

The basic principle of screening recombinant DNA libraries is that bacteriophage plaques, or bacterial colonies containing plasmids or cosmids, contain relatively large amounts of insert DNA that can be detected either directly by hybridization (see below) or indirectly by the protein that may be expressed from the cloned segment (UNIT 6.7). The first step in the nucleic acid hybridization screening procedure is to grow large numbers of colonies or plaques on agar plates. Replica copies of these colonies are transferred to nitrocellulose filters, where they can be screened. In this section the techniques for producing large numbers of colonies and plaques, and for transferring these to filter membranes, are discussed. Prerequisites to these procedures are that the library must already be chosen and the number of clones to be screened must be determined (see introduction to this chapter).

Plating and Transferring Bacteriophage Libraries

UNIT 6.1

Bacteriophage are plated onto agar plates at high density so that as many as 1 million different plaques can be screened. The bacteriophage plaques are then transferred to nitrocellulose filters, denatured, and baked. The library and the number of clones to be screened are predetermined. Principles for choosing the plaque density and the number of plates to be used are outlined in the commentary.

BASIC PROTOCOL

Materials

Host bacteria, selection strain if applicable (UNIT 1.10; Table 1.4.5; Table 5.10.1)
Recombinant phage (UNIT 5.10)
0.7% top agarose (prewarmed; UNIT 1.1)
82-mm or 150-mm LB plates; or 245 × 245-mm Nunc bioassay LB plates (UNIT 1.1)
0.2 M NaOH/1.5 M NaCl
0.4 M Tris·Cl, pH 7.6/2× SSC
2× SSC (APPENDIX 2)
Nitrocellulose membrane filters (or equivalent)
20-G needle
46 × 57-cm Whatman 3MM or equivalent filter paper
80°C vacuum oven or 42°C oven

Plating bacteriophage

1. Determine the titer of the library by serial dilution as described in UNITS 1.11 & 5.7.

For λ vectors that allow genetic selection against nonrecombinants, plating should be done on the appropriate bacterial strain (e.g., P2 lysogen for EMBL vectors). LB plates should be poured several days in advance to allow them to dry prior to plating. The large Nunc plates are particularly prone to condensation on the surface of the agar, but this can be alleviated by allowing them to sit on the benchtop with covers removed for a few minutes to several hours before use.

2. Mix recombinant phage and plating bacteria (prepared as described in UNIT 1.11) in a culture tube as outlined in Table 6.1.1 and incubate 20 min at 37°C.
3. Add 0.7% top agarose to culture tube and transfer mixture to LB plates. Disperse bacteria and agarose on plates by tilting the plates back and forth. Mix cells and agarose for the large Nunc plates by gently inverting several times in a capped 50-ml tube prior to plating.

Screening Recombinant DNA Libraries

6.1.1

Top agarose rather than top agar should be used as agar tends to lift off with the nitrocellulose filter.

Melt the top agarose and cool to 45° to 50°C before use. If top agarose is too hot it will kill the bacteria, while if it is too cold the library will solidify in the tube.

4. Incubate plates at 37°C until plaques cover the plate but are not confluent. Incubation time varies between 6 and 12 hr and depends on type of phage and bacteria used. Store at 4°C.

Do not incubate unattended overnight, but rather place at 4°C and allow to continue growth the next day. Allowing phage plaques to incubate for the correct amount of time is critical. The object is to optimize two parameters. First, the plaques must be large enough to contain sufficient DNA to give a good signal. Second, if the plaques are too large and become confluent they are difficult to purify in subsequent steps. Because most nucleic acid probes give a very strong signal, we tend to prefer having smaller plaques and weaker signals.

5. Incubate plates at 4°C for at least 1 hr before applying filters.

Transferring to nitrocellulose filters

6. Label nitrocellulose filters with a ballpoint pen and apply face down (ink side up) on cold LB plates bearing bacteriophage plaques. This is best accomplished by touching first one edge of the filter to the agarose and progressively laying down more of the filter as it wets. Bubbles should be avoided. If difficulties are encountered the filter should not be adjusted on the plate, but rather removed and replaced with a new filter.

Nitrocellulose filters should be handled only with forceps or gloved hands.

7. Leave filters on plates for 1 to 10 min to allow transfer of phage particles to the filter. During this transfer period the orientation of the filter to the plate is recorded by stabbing a 20-G needle through the filter into the agar at several asymmetric points around the edge of the plate. Up to five replicas can be made from each plate. Remove the filter slowly from the plate with blunt, flat forceps and place face up on paper towels or filter paper.

Some investigators dip the needle used to orient the filter in India ink to more clearly mark the filter and agar. Other investigators mark the back of the agar plate with a black marker.

Making two replicas from each filter, hybridizing both to the DNA probe, and comparing the autoradiographs of the replica filters eliminates many possible artifacts. This is particularly helpful when screening with an oligonucleotide probe.

8. Dry the filters on the benchtop for at least 10 min.

This drying process binds the plaques to the filter.

Table 6.1.1 Recommended Mixtures for Plating Bacteriophage Libraries

LB plate ingredient	Plate size		
	82 mm	150 mm	245 × 245 mm ^a
Bacteria ^b (ml)	0.2	0.5	2
Phage, pfu	5,000	20,000-30,000	150,000
Top agarose, ml	3	7	30

^aNunc Bioassay plates distributed by Vanguard International.

^bPlating bacteria are prepared as described in Chapter 1.

Denaturation and baking

9. Place 46 × 57-mm Whatman 3MM paper on the benchtop and saturate with 0.2 M NaOH/1.5 M NaCl. Place filters on the paper face up for 1 to 2 min.

The 3MM paper should be wet enough to allow immediate saturation of the filters, but not so wet that the solution pools on the surface.

10. Transfer filters (face up) to 3MM paper saturated with 0.4 M Tris·Cl, pH 7.6/2× SSC for 1 to 2 min and then to 3MM paper saturated with 2× SSC for 1 to 2 min.

Some investigators immerse the filters in all three solutions. This procedure can make the plaques detected by hybridization appear diffuse.

11. Dry filters in a vacuum oven 90 to 120 min at 80°C or overnight in a regular oven at 42°C. Store at room temperature in folded paper towels or other absorbent paper until needed for hybridization (described in UNIT 6.3 or 6.4).

COMMENTARY

Background Information

There are two parts to this protocol—plating the library and preparing filters. The number of bacteriophage per plate determines the number of plates that must be poured. This number is defined by the number of recombinants in the library (i.e., base of the library) and the frequency of the expected clone in the library. There is no advantage to screening more than 3 to 5 times the base of the library. The frequency of the clone in the library is determined as follows.

cDNA libraries: the expected frequency of the desired RNA among the total RNA of the cell, ranging from $1/100$ to $1/50,000$.

Genomic libraries: the size of the insert divided by the total genome size.

Subgenomic libraries: the size of insert per total genome size times the fold purification of the DNA fragment (usually 10- to 50-fold).

The usefulness of a recombinant phage library depends on the ability to screen a large number of phage and identify the clone that carries the DNA sequence of interest. This has been made possible by the technique of in situ plaque hybridization described by Benton and Davis (1977). The phage are allowed to multiply in host bacteria in a thin layer of agarose on regular bacterial plates. When nitrocellulose is applied to the agarose, phage particles and unpackaged DNA adsorb to the filter to produce a replica of the plate surface. If the agarose surface is not excessively wet, there will be little spreading of the phage on the filter. Subsequent treatment of the filter with sodium hydroxide destroys the phage particles and denatures the phage DNA which then binds to the nitrocellulose. Neutralization of the filters is required to maintain the integrity of the nitrocellulose. Hy-

bridization of these filters to a DNA or RNA probe will identify the location of the phage plaque of interest, which can then be recovered from the plate.

A common variation of this technique is the substitution of one of the nylon-based membranes for nitrocellulose (see UNIT 2.9). The advantage of nylon membranes is their durability, which allows multiple hybridizations to the same filter and allows one to sequentially clone several genes from the same library using a single set of filters. However, nylon filters do not offer an improvement in sensitivity and are often more expensive than nitrocellulose filter paper.

Literature Review

The molecular basis of λ phage replication and the adaptation of the λ genome for molecular cloning has been reviewed by Arber et al. (1983) and Williams and Blattner (1980). Principles governing the plating of λ phage have been outlined by Arber (1983); see also UNIT 1.10. Thorough understanding of these principles has led to a universal approach to plating phage libraries.

Critical Parameters

To prevent recombination between different phage, do not allow them to overgrow, and grow them in recombination-minus hosts where possible. Calculations of the amount of phage stock to be used per plate should be based on a recent titration, and plating cells should be fresh.

Filters must not become brittle during this procedure; brittle filters will be destroyed during the hybridization process. This can be avoided by limiting the time in the hydroxide solution to less than 5 min, making certain that

the 0.4 M Tris-Cl, pH 7.6/2× SSC brings the filters to neutral pH, and limiting the baking to 2 hr.

Troubleshooting

Plaques should be visible on the plate before filters are made. If there appears to be poor bacterial growth, it is possible that the top agarose was too warm and many bacteria were killed, or that the phage titer was higher than expected and most host cells were lysed. Lower than expected phage titer could be due to an inaccurate titration of the phage stock, poor host-cell preparation, or too little time for adsorption.

The preparation of the nitrocellulose filters will only be tested after hybridization is complete. Occasionally, hybridization to a plaque will produce a streak instead of a discrete circle on the autoradiograph, making location of the correct plaque difficult. Steps that will often correct this problem include: (1) drying plates with the cover removed for 1 to 2 hr before applying the filter, (2) drying the filters well before the hydroxide treatment, and (3) making certain that the face (phage side) of the filters is not directly in contact with the solutions.

Anticipated Results

This plating procedure characteristically produces plates with an even distribution of dense phage particles. It is sensitive enough to allow identification of a phage by hybridization even when the phage are plated at high density (>5000 plaques per 82-mm plate). A signal is easily visible after 18 to 24 hr, when filters are hybridized to a nick-translated DNA probe with activity of $>10^7$ counts/ μ g DNA.

Time Considerations

Usually plaques will become visible within 6 to 10 hr after plating. Bacteriophage should generally not be allowed to grow longer than necessary to visualize the plaques. Using the procedure outlined, even a large number of filters can be processed in a single day.

Literature Cited

- Arber, W. 1983. A beginner's guide to lambda biology. *In* Lambda II (R.W. Hendrix, J.W. Roberts, F.W. Stahl, and R.A. Weisberg, eds.) pp. 381-395. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Arber, W., Enquist, L., Hohn, B., Murray, N., and Murray, K. 1983. Experimental methods for use with lambda. *In* Lambda II (R.W. Hendrix, J.W. Roberts, F.W. Stahl, and R.A. Weisberg, eds.) pp. 433-466. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Williams, B.G. and Blattner, F.R. 1980. Bacteriophage lambda vectors for DNA cloning. *In* Genetic Engineering, Vol. 2 (J.K. Setlow and A. Mullander, eds.) p. 201. Plenum, NY.

Key References

- Benton, W.D. and Davis, R.W. 1977. Screening λ gt recombinant clones by hybridization to single plaques in situ. *Science* 196:180-182.

Describes the method of plaque hybridization developed by the authors to allow isolation of phage possessing specific cloned DNA sequences.

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Plating and Transferring Cosmid and Plasmid Libraries

UNIT 6.2

BASIC
PROTOCOL

A bacterial suspension is suctioned through a porous membrane, leaving the bacteria bound to the membrane surface. The membrane is transferred, bacteria up, to an agar plate upon which the bacteria will receive enough nutrients to grow into colonies. These filters can then be used for replica platings and for hybridization with specific DNA probes.

Materials

LB plates containing antibiotic (UNIT 1.1)
LB medium (UNIT 1.1)
LB plates containing 50 µg/ml chloramphenicol (UNIT 1.1)
0.5 M NaOH
1 M Tris-Cl, pH 7.5
0.5 M Tris-Cl, pH 7.5/1.25 M NaCl
10- or 15-cm Whatman 3MM or equivalent filter paper discs
Sintered glass filter with vacuum
Nitrocellulose membrane filters (10- or 15-cm, Millipore HATF)
20 × 20-cm Whatman 3MM or equivalent filter paper
20 × 20-cm glass plate
20-G needle
46 × 57-cm Whatman 3MM or equivalent filter paper
80°C vacuum oven

NOTE: All materials coming into contact with *E. coli* must be sterile.

Plating cosmids

1. Start with plasmid or cosmid library produced after transformation, transfection, or amplification (UNIT 5.7).
2. Determine titer of the library by serial dilutions using plates containing antibiotics (see UNIT 1.3).

Remaining library suspension can be held at 4°C overnight with only minimal loss of viable bacteria.

A 10-cm nitrocellulose filter can accommodate 10,000 to 20,000 colonies, while a 15-cm filter can hold up to 50,000.

3. Calculate the appropriate amount of the bacterial suspension for plating and dilute the suspension in LB medium such that there is the desired amount of bacteria in 5 ml (10-cm filter) or 10 ml (15-cm filter) of solution.
4. Meanwhile, prepare a layer of 10- or 15-cm Whatman 3MM paper discs on either the bottom part of a sintered glass Buchner funnel or on a porcelain filter funnel. Pour 10 to 20 ml LB medium over two or three layers of 3MM paper discs to make a level bed. The same pad of discs can be used for many filters.

Sterilize filter apparatus and filter paper before use. The 3MM and nitrocellulose filters can be sterilized by autoclaving them while wrapped in aluminum foil.

The purpose of this step is to spread the bacteria uniformly across the surface of a nitrocellulose filter. The filtering apparatus must be level, it must create a uniform suction to all the surface of the filter, and it should be easy to move the filters to and from the apparatus.

Screening
Recombinant
DNA Libraries

6.2.1

5. Label a nitrocellulose filter with a ballpoint pen on the side opposite that where the bacteria will be plated. Place the filter on the surface of the LB/antibiotic plate to wet it.

The antibiotic plate must be permissive for cosmid- or plasmid-bearing bacterial cells and usually is ampicillin or tetracycline.

Most ballpoint pen inks do not smudge during the hybridization reaction. If the one you choose runs, try another type.

6. Remove the wet filter from an antibiotic plate to the filtration apparatus.

The suction should be off.

Carefully pipet the 5 to 10 ml of bacterial suspension onto the surface of the nitrocellulose filter, leaving the outer 4 to 5 mm of the filter free of solution.

This outside bacteria-free ring leaves enough surface area to work with the filter without smearing or losing the colonies.

7. Slowly suction the solution down through the filter, taking care not to create any preferential suction pockets that would concentrate the bacteria. After suctioning all of the solution through the filter, transfer the filter back to the antibiotic plate on which it was wetted.

In laying the filter down on the agar surface, take care to avoid trapping any air bubbles between the surface of the plate and the filter.

8. Plate the entire library in this way and incubate the plates upside down (agar side up) at 37°C until the colonies are ~1 mm in diameter.

Do not overgrow the filters, as smaller colonies can be lost beneath larger, faster-growing recombinant bacteria.

Preparing replica filters

9. Label and wet another set of nitrocellulose filters, as described in step 5.

10. Remove the initial library filter from its plate and place on several sheets of 20 × 20 cm 3MM paper, bacteria side up. While wearing gloves, carefully position the wetted replica filter above the bacterial lawn. Lay the second filter upon the first, leaving the two filters offset by 2 to 3 mm.

This overlap will help in the separation of the two filters after the replica transfer.

Do not allow air bubbles to form between the two filters. These are excluded by touching the second filter to the first in the middle and then allowing the edges to fall.

11. Lay three sheets of 20 × 20-cm 3MM paper on the two filters, followed by a 20 × 20 cm glass plate. Using the palms of your hands, press with all your weight down on the glass plate, thus transferring the bacterial colonies from the library filter to the replica filter.

12. Remove the glass plate and the filter paper and, using a 20-G needle, punch holes 2 to 4 cm apart through both of the filters. These holes will allow the orientation of the film produced from the replica filter down on the library filter for the isolation of the correct clones.

13. Carefully peel the two filters apart, placing them both bacteria up, on their respective agar plates. Grow the replica colonies at 37°C overnight, leaving the library filters at 25°C overnight. After overnight growth, store the library filters on the agar plates at 4°C, while screening the replica filters.

Multiple replica filters can be made from the same library filter. Incubate library filters 2 to 4 hr at 37°C or overnight at 25°C to allow regrowth of the colonies.

Then repeat steps 9 to 13. Normally, two copies of the cosmid are hybridized to each probe.

14. After the bacterial colonies have grown, the cosmids or plasmids on the replica filter are amplified by transferring them to an LB plate containing 50 µg/ml chloramphenicol and incubating at 37°C for 4 to 10 hr. This step will increase the signal produced by hybridization.

Preparing filters for hybridization

15. Remove the replica filters from the LB/chloramphenicol plates, place filters bacteria side up on a sheet of 46 × 57-cm 3MM paper soaked with 0.5 M NaOH, and leave them for 5 min.
16. Carefully transfer to a sheet of 46 × 57-cm 3MM paper soaked with 1 M Tris-Cl, pH 7.5. Allow neutralization to occur for 5 min.
17. Transfer to a third 46 × 57-cm filter soaked in 0.5 M Tris-Cl, pH 7.5/1.25 M NaCl. Neutralize 5 min.
18. Transfer filter to a dry sheet of 3MM paper to allow filter to dry.

After filters are completely dry, stack them on paper towels or other adsorbent paper. Each nitrocellulose filter should be separated by paper towels from other filters.

19. Transfer the stacked filters to a vacuum oven at 80°C for 90 min. Remove filters and hybridize with a nick-translated probe, as described in *UNITS 6.3* and *6.4*.

COMMENTARY

Background Information

There are two commonly used protocols for the screening of recombinant bacteria with hybridization probes. The first method involves the spreading of bacteria on the surface of agar using a sterile spreader (*UNIT 1.3*). A nitrocellulose membrane filter is then placed on top of the colonies and most of each colony is transferred to the filter. The filter is then treated as described in steps 15 to 19. This method works well when relatively small numbers of positive colonies are being selected (up to several thousand).

The second method employs a matrix of some type (here nitrocellulose filters are used) upon which bacteria can be plated and grown when the filter is placed on top of a nutrient agar surface. Once the plated bacteria have grown into visible colonies, the filters can be used for replica plating and in situ hybridization analysis.

Critical Parameters

In order to provide a uniform lawn of recombinant bacteria for screening, it is critical to ensure that the suction applied to the filters is uniform and not spotty. The best way to accomplish this is to suction the suspension through

the filter slowly and to avoid any preferential suction sites in the filter. Make sure that the apparatus is level and that adequate layers of LB-soaked chromatography paper are used. Air bubbles will prevent bacterial growth, so be certain that air is not trapped between the filter and the agar surface.

Time Considerations

Once the apparatus is set up, it takes ~5 min per filter to wet the filter, suction the bacteria, and transfer to an LB plate. The colonies take ~15 hr to grow at 37°C, after which they can be transferred to 4°C until ready for the replica platings. Replica plating also requires 5 min per filter, and resulting filters will be ready for denaturation and hybridization after 15 hr at 37°C.

Key Reference

Hanahan, D. and Meselson, M. 1983. Plasmid screening at high density. *Meth. Enzymol.* 100:333-342.

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PURIFICATION OF BACTERIOPHAGE, COSMID, AND PLASMID CLONES

SECTION III

After the screening procedure has identified potentially desirable clones they must be purified. The following protocols (which are similar to those in *UNITS 1.3* and *1.11*) minimize the number of steps because of the large number of clones on a plate and because of the difficulties associated with retesting these clones.

Purification of Bacteriophage Clones

UNIT 6.5

BASIC PROTOCOL

Phage plates are correctly oriented to the autoradiograph film, and a region that should contain the clone of interest is sampled by toothpicking each phage plaque onto secondary plates containing a lawn of host cells. Alternatively, a plug of agarose can be taken from the primary plate, placed in SM, and this solution used to plate a small secondary library. Plaques on the secondary plates are transferred to nitrocellulose filters, hybridized to ^{32}P -labeled probe, and an isolated positive plaque is picked, diluted in SM, and regrown. This process is repeated until the desired plaque is purified.

Materials

- 0.7% top agarose (*UNIT 1.1*)
- Host bacteria (OD_{600} 1.5 to 2 in 10 mM MgSO_4)
- LB plates (*UNIT 1.1*)
- Suspension medium (SM; *UNIT 1.11*)
- Chloroform
- Sterile round toothpicks (*UNIT 1.1*) or Pasteur pipet
- Nitrocellulose membrane filters
- Additional reagents and equipment for autoradiography (*APPENDIX 3*) and phage titering (*UNIT 1.11*)

Growth of secondary plaques

1. Plate 3 ml of 0.7% top agarose containing 200 μl host bacteria on 82-mm LB plates (one plate per clone) and allow to set 10 min.

Top agar cannot be used because it tends to lift off with the nitrocellulose filter.

2. Orient the autoradiograph to the primary library filters by radioactive tags that have been placed on the material used to support the filters. Then mark the autoradiograph at the points where the filters contain needle holes. Place plates containing the library on top of the autoradiograph on an X-ray view box and orient according to the needle marks.

To reduce the number of irrelevant clones, it is often helpful to produce two different exposures of the primary filters. One can then eliminate from consideration spots that do not appear on both autoradiographs.

- 3a. Insert toothpicks first into the primary plate in the area over the hybridization spot on the autoradiograph and then into the top agarose of one of the secondary plates prepared in step 1 above (see *UNIT 1.11*). A grid is helpful to guide spacing of the stabs 5 to 8 mm apart on the secondary plate. Initially attempt to pick individual plaques from the primary plate, then insert the toothpicks at random to ensure that an entire circular area with a 1-cm diameter has been well sampled. This usually requires 30 to 40 stabs per potential clone. It is advisable to make a duplicate secondary plate at the time of picking by simply sticking each toothpick into a second secondary plate in roughly the same pattern as the first.

Screening
Recombinant
DNA Libraries

6.5.1

- 3b. An alternate screening procedure to the above is as follows. Insert the large end of a Pasteur pipet into the top agarose of the primary plate to cut a circular plug corresponding to the region of the autoradiogram demonstrating radioactivity. Remove this plug with the Pasteur pipet or with a spatula and place into 1 ml SM with one drop of chloroform. Allow to sit 1 to 2 hr and then titer. As soon as the titer is known, make 3 to 6 plates with a density of <500 phage per plate. These secondary plaques are handled as in step 4 below.

Screening secondary plaques by hybridization

4. Grow secondary plates at 37°C overnight. Transfer plaques to nitrocellulose filters, process, hybridize, wash, and expose as outlined in *UNITS 6.1 to 6.4*. Mark filter orientation points on the autoradiograph and identify positive plaques on the secondary plates. Insert toothpick into the most strongly hybridizing plaque for each clone and placed into 1 ml SM for 5 min. Plate 1 µl of this phage stock and 1 and 10 µl of a 1:100 dilution onto tertiary LB plates.
5. Screen tertiary plates as above. Insert toothpick into an isolated hybridizing plaque and transfer to SM. This phage stock may be pure, but it is plated and evaluated by hybridization of these plates. If all plaques are positive, make a final SM stock from one of the plaques on these plates. Repeat these steps until the phage is pure. A high-titer stock solution can then be made, as outlined in *UNIT 1.12*.

COMMENTARY

Background Information

Careful purification of the clone of interest away from contaminating phage is required before growth and characterization of the clone can proceed. It is common for a "purified" clone to be contaminated by a second phage, leading to confusing results and wasted time. Several rounds of purification should be performed even if the phage appears pure as early as the secondary screening stage. This approach is similar to that presented by Kaiser and Murray (1984).

Critical Parameters

Plaque purification depends on the ability to go from a hybridization signal on the autoradiograph back to the correct plaque on the LB plate. Thus, attention to orientation of the filter to plate, filter to X-ray film, and film to plate is important.

If more than one potential clone is being plaque purified, it is important to prevent cross-contamination (e.g., via SM or agarose) between clones. Because only a single plaque is sampled at each step of purification, contamination could result in the loss of clones.

Troubleshooting

Occasionally, a clone will be lost during the purification process. When this happens it is usually best to go back as far as possible, i.e., to the secondary plates or to the SM stock.

Potential clones lost with the toothpick technique (step 3a) can sometimes be recovered by the plug procedure (step 3b). It is rarely worthwhile to attempt to purify a clone more than twice, since two unsuccessful attempts usually indicate a false primary clone.

Anticipated Results

This technique normally recovers >90% of clones confirmed by duplicate filters.

Time Considerations

A few hours are required every day for 5 to 8 days. Days picking and plating positive phage can alternate with days setting up filters and hybridization. Maintaining this schedule requires washing filters and obtaining an autoradiograph in 1 day.

Literature Cited

Kaiser, K. and Murray, N.E. 1984. The use of phage lambda replacement vectors in the construction of representative genomic DNA libraries. In *DNA Cloning: A Practical Approach*, Vol. 1 (D.M. Glover, ed.) pp. 1-47. IRL Press, Oxford.

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Purification of Cosmid and Plasmid Clones

Cosmid- or plasmid-bearing colonies that are identified by hybridization are purified by spreading the cosmids or plasmids on an agar plate and repeating the colony hybridization.

Materials

Cold LB medium containing antibiotic (UNIT 1.1)

LB plates containing antibiotic (UNIT 1.1)

Round toothpicks (UNIT 1.1)

Nitrocellulose membrane filters

Spreader (UNIT 1.3)

Additional reagents and equipment for plating bacteria (UNIT 1.3), plating and transferring plasmid/cosmid libraries (UNIT 6.2), plasmid minipreps (UNIT 1.6), and autoradiography (APPENDIX 3)

NOTE: All materials coming into contact with *E. coli* must be sterile.

1. Pick the positive clones, as detected by the in situ hybridization of nitrocellulose replica filters (UNITS 6.3 & 6.4), with a sterile toothpick.

If plate is dense with colonies, be sure to pick from a 3- to 5-mm circle to ensure selecting the correct clone.

2. Rinse the tip of the toothpick off into a microcentrifuge tube containing 1 ml cold LB medium with the appropriate antibiotic. Store these tubes at 4°C to inhibit any continued growth.

The vector must encode a gene conferring resistance to the appropriate antibiotic.

3. Plate out, using a sterile spreader, from 1 to 25 μ l of the bacterial suspension onto an LB plate with the appropriate antibiotic. The correct number of clones to screen is from 25 to 250 per 100-mm plate. Allow the colonies to grow overnight at 37°C.
4. Make a replica copy of the bacterial lawn onto a nitrocellulose filter (UNIT 6.2). Denature, renature, bake, and hybridize as described in UNIT 6.2.
5. From the autoradiograph of the secondary plate, select the most isolated, positive colony. Grow the colony and isolate the DNA (UNIT 1.3).

COMMENTARY

Critical Parameters

If the colonies are too dense, purification of a single colony following a second round of hybridization is difficult. If the colonies are too sparse, many plates must be screened to identify a single hybridizing plaque. The number of clones required depends upon the original number of colonies picked in the original toothpicking. The purpose of rinsing the tip of the toothpick in cold LB medium and keeping the suspension cold is to stop the overgrowth of a single colony and to be able to reliably predict the titer of the suspension.

Anticipated Results

Plating cosmid- or plasmid-bearing bacteria on an agar surface at the appropriate density—

from 25 to 250 colonies per 100-mm plate—will allow the isolation of a single positive clone.

Time Considerations

Starting from a positive colony identification, this procedure requires one night for the colonies to grow, 10 min per plate for colony transfer to nitrocellulose, and one night for the second hybridization. If a probe with a high specific activity is used, an autoradiograph can be produced from the washed filters in a few hours.

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Oligonucleotide-Directed Mutagenesis without Phenotypic Selection

UNIT 8.1

BASIC PROTOCOL

A DNA sequence can be specifically altered by synthesizing the desired sequence change within an oligonucleotide, and then converting this into a biologically active circular DNA strand by using the oligonucleotide to prime in vitro synthesis on a single-stranded circular template. This protocol uses a DNA template containing a small number of uracil residues in place of thymine, as shown in Figure 8.1.1 (see further commentary on p. 8.1.5). Use of the uracil-containing template allows rapid and efficient recovery of mutants; in principle this same template can be applied to most of the other mutagenesis protocols in use.

Materials

Single-stranded bacteriophage vector with insert
TY medium containing 0.25 µg/ml uridine (UNIT 1.5)
E. coli CJ236 or alternative *dur⁻ ung⁻ F'* strain (Invitrogen and Table 1.4.5)
5× PEG/NaCl solution
TE buffer (APPENDIX 2)
T4 polynucleotide kinase (UNIT 3.10) and 10× kinase buffer (UNIT 3.4)
10 mM ATP (UNIT 3.4)
Mutagenic oligonucleotide primer
100 and 500 mM EDTA, pH 8.0 (APPENDIX 2)
20× SSC (APPENDIX 2)
5× polymerase mix
T4 OR T7 DNA polymerase (*not* Sequenase; see UNIT 3.5)
T4 DNA ligase (measured in Weiss units; UNIT 3.14)

Additional reagents and equipment for phage titering (UNIT 1.11), phenol extraction (UNIT 2.1), ethanol precipitation (UNIT 2.1), agarose gel electrophoresis (UNIT 2.5), preparation and transfection of competent cells (UNIT 1.8), and DNA sequence analysis (UNIT 7.4)

Preparing uracil-containing DNA template

1. With a sterile Pasteur pipet, remove one plaque produced by single-stranded phage containing the DNA of interest and place in 1 ml sterile TY medium in a 1.5-ml microcentrifuge tube.
2. Incubate 5 min at 60°C to kill cells, vortex vigorously to release phage from the agar, then pellet cells and agar with a 2-min spin in microcentrifuge.
3. Place 100 µl of resulting supernatant into 1-liter flask containing 100 ml TY medium supplemented with uridine to 0.25 µg/ml; add 5 ml of a midlog culture of *E. coli* CJ236 (*dur⁻ ung⁻ F'*) (Kunkel et al., 1987).

*These proportions result in a multiplicity of infection of considerably less than one per cell. Thus, essentially all of the input phage infect cells and are "passaged" through the *dur⁻ ung⁻* strain. Since few uracil-lacking phage remain, a single cycle of growth results in a sufficient survival difference (as measured by titers on *ung⁺* and *ung⁻* hosts) to make the DNA suitable for the in vitro mutagenesis protocol. Neither the thymidine nor the adenosine supplementation of medium originally described (Kunkel, 1985) is necessary.*

4. Incubate with vigorous shaking at 37°C for 6 to 18 hr.

Good aeration provided by vigorous shaking is important for high-phage titers.

Mutagenesis of Cloned DNA

8.1.1

Supplement 13

5. Pellet the cells by centrifugation at $5000 \times g$ for 30 min. The clear supernatant should contain phage at a titer of $\sim 10^{10}$ to 10^{11} pfu/ml.

The phage yield may vary, depending on the vector and strain used. For example, experience with E. coli RZ1032 for growth of M13 derivatives containing amber mutations suggests that phage titers of 2 to 5×10^{10} pfu/ml are not unusual.

6. Titer the phage on any *E. coli ung*⁻ (e.g., CJ236) versus *ung*⁺ strain (e.g., JM105, JM107, or JM109). Phage containing uracil in the DNA have normal biological activity in the *ung*⁻ host, but $>100,000$ -fold lower survival in the *ung*⁺ host.
7. Precipitate phage by adding 1 vol of 5× PEG/NaCl solution to 4 vol supernatant. Mix and incubate 1 hr at 0°C.
8. Collect precipitated phage by centrifugation at $5000 \times g$ for 15 min. Drain the pellet well, resuspend in 5 ml TE buffer in a 15-ml Corex tube, and vortex vigorously.
9. Place the resuspended phage solution on ice for 1 hr and then centrifuge as above.

This step removes residual cell debris and has proven useful in reducing the level of endogenous low-molecular-weight DNA, which can nonspecifically prime in subsequent in vitro DNA polymerase reactions.

10. Purify the single-stranded phage DNA by phenol extraction and ethanol precipitation as described in UNIT 2.1. Determine the DNA concentration spectrophotometrically at 260 nm ($1 \text{ OD}_{260} = 36 \mu\text{g/ml}$).

Normally, further purification of the DNA is unnecessary in order to achieve high efficiencies of in vitro mutagenesis. If problems related to template purity are encountered or if mutant production approaching 100% is needed, the DNA can be subjected to any standard purification procedure, since the substitution of a small percentage of thymidine residues by deoxyuridine will not affect the physical properties of the DNA.

*In principle, any cloning vector that can be passaged through an *E. coli dut*⁻ *ung*⁻ strain can be used with the uracil selection technique. Once the uracil-containing DNA is prepared, it can be used as a standard template in a variety of in vitro methodologies for altering DNA sequences (Smith, 1986). Presented below is a typical oligonucleotide-directed mutagenesis experiment.*

Primer extension and product analysis

11. To a 1.5-ml microcentrifuge tube add the following:

2 μl 10× T4 polynucleotide kinase buffer
2 μl 10 mM ATP
Mutagenic oligonucleotide (15 to 50 nucleotides long)
H₂O to 20 μl

The amount of oligonucleotide used depends upon the desired molar ratio of oligonucleotide to single-stranded template. Ratios from 2:1 to 10:1 are routine, but in some instances higher ratios may be useful. For primers that are 15 to 20 bases in length, this corresponds to 4 to 30 ng/ μg single-stranded M13 template.

If the oligonucleotide has already been phosphorylated, proceed directly to step 13.

12. Add 2 U of T4 polynucleotide kinase and incubate 60 min at 37°C. Terminate the reaction by adding 3 μl of 100 mM EDTA, and heat to 70°C to denature the enzyme.

Phosphorylation of the oligonucleotide is required for the subsequent ligation step.

13. To the phosphorylated oligo (20 μ l) add the single-stranded circular uracil-containing DNA template (typically 1 μ g in 1 μ l); add 1.25 μ l of 20 \times SSC.
14. Mix thoroughly, spin 5 sec in microcentrifuge, then place tube in 500-ml beaker of water at 70°C. Allow to cool to room temperature. After another 5-sec spin to collect condensation, place tube on ice.

The precise hybridization conditions depend upon the stability of the heteroduplex formed between the mutagenic oligonucleotide and the template DNA. The ability of the oligonucleotide to prime in vitro DNA synthesis efficiently and at the appropriate position under the chosen hybridization conditions can be determined by DNA sequence analysis.

15. To the hybridization mixture add the following:

20 μ l 5 \times polymerase mix
 2.5 U T7 or T4 DNA polymerase
 2 U T4 DNA ligase
 H₂O to 100 μ l

Since the T4 DNA polymerase activity in commercial preparations can vary with source and age of the enzyme, it may be necessary to add more than 2.5 U.

Enzymes are added last. T7 or T4 DNA polymerase is preferred over Klenow fragment because it will not "strand displace" the mutagenic oligonucleotide after synthesis is completed; this permits efficient ligation and expression of the mutation. T7 DNA polymerase (not Sequenase) is ideal for complete synthesis due to its high processivity. With some oligonucleotide template combinations, this enzyme does not work well, in which case 1 U Klenow fragment can be substituted.

16. Mix thoroughly, then incubate 5 min at 0°C, 5 min at room temperature, and 2 hr at 37°C.

The reaction is begun at lower temperatures to polymerize a small number of bases onto the 3' end of the oligonucleotide, thus stabilizing the initial duplex between the template DNA and the mutagenic oligonucleotide primer. However, since T4 DNA polymerase does not utilize long stretches of single-stranded DNA template well at low temperature, synthesis is then completed at 37°C. The use of a high concentration of dNTPs (500 μ M each) serves to optimize DNA synthesis and to reduce the 3'-exonuclease activity of the T4 DNA polymerase.

17. Add 3 μ l of 500 mM EDTA to terminate the reaction.
18. Analyze 20 μ l of the reaction mixture (200 ng of DNA) by agarose gel electrophoresis in a 0.8% agarose gel. For comparison, adjacent lanes should contain the following standards: single-stranded circular viral DNA; double-stranded, closed circular DNA (RFI); and nicked double-stranded circular DNA (RFII).

A successful reaction should convert essentially all the single-stranded template DNA into RFI and RFII DNA. In the absence of strand displacement (e.g., using T4 DNA polymerase), both RFI and RFII products yield mutations at high efficiency.

Transfection and DNA sequence analysis

19. Based upon an estimate from the gel analysis, use 1 to 100 ng of double-stranded DNA product to transfect any desired *ung*⁺ strain of *E. coli* cells.
20. Resulting clones (as phage plaques or colonies) can be selected or, if no phenotype is known, chosen randomly for isolation of pure genetic stocks. These can be analyzed by sequencing the DNA.

REAGENTS AND SOLUTIONS

5× PEG/NaCl solution

15% (wt/vol) polyethylene glycol 8000
2.5 M NaCl

5× polymerase mix

100 mM Tris-Cl, pH 8.0
10 mM dithiothreitol
50 mM MgCl₂
2.5 mM each of dATP, dTTP, dGTP, dCTP
5 mM ATP

Use of highly purified (e.g., by HPLC) dNTPs is critical.

COMMENTARY

Background Information

The basis of this method is use of a DNA template containing a small number of uracil residues in place of thymine (Fig. 8.1.1). The uracil-containing DNA is produced within an *E. coli dur⁻ ung⁻* strain. *Escherichia coli dur⁻* mutants lack the enzyme dUTPase and therefore contain elevated concentrations of dUTP which effectively competes with TTP for incorporation into DNA. *Escherichia coli ung⁻* mutants lack the enzyme uracil *N*-glycosylase which normally removes uracil from DNA. In the combined *dur⁻ ung⁻* mutant, deoxyuridine is incorporated into DNA in place of thymidine and is not removed. Thus, standard vectors containing the sequence to be changed can be grown in a *dur⁻ ung⁻* host to prepare uracil-containing DNA templates for site-directed mutagenesis.

For the in vitro reactions typical of site-directed mutagenesis protocols, uracil-containing DNA templates are indistinguishable from normal templates. Since dUMP in the template has the same coding potential as TMP, the uracil is not mutagenic, either in vivo or in vitro. Furthermore, the presence of uracil in the template is not inhibitory to in vitro DNA synthesis. Thus, this DNA can be used in vitro as a template for the production of a complementary strand that contains the desired DNA sequence alteration, but contains only TMP and no dUMP residues (Fig. 8.1.1).

After completing the in vitro reactions, uracil can be removed from the template strand by the action of uracil *N*-glycosylase. Glycosylase treatment can be carried out with purified enzyme, but it is most easily achieved by introducing the unfractionated products of the in vitro incorporation reaction into wild-type (i.e., *ung⁺*) *E. coli* cells. Treatment with the glycosylase, either in vitro or in vivo, releases uracil,

producing apyrimidinic (AP) sites in the template strand. These AP sites are lethal lesions, presumably because they block DNA synthesis and are sites for incision by AP endonucleases which produce strand breaks.

Thus, the template strand is rendered biologically inactive and the majority of progeny arise from the infective complementary strand which contains the desired alteration (Fig. 8.1.1). The resulting high efficiency of mutant production (typically >50%) allows one to screen for mutants by DNA sequence analysis, thus identifying mutants and confirming the desired alteration in a single step. This feature is particularly advantageous when no selection for the desired mutants is available.

Literature Review

Several variations of in vitro mutagenesis by primer extension that yield mutants with high efficiency have been developed (for review, see Smith, 1986). The procedure described here is the simplest site-directed mutagenesis protocol, but applied to a special uracil-containing template which allows rapid and efficient recovery of mutants (Kunkel, 1985; Kunkel et al., 1986). In principle, this same template can be applied to most of the other protocols in use.

Critical Parameters

Highly efficient mutagenesis depends upon a good template and a successful DNA polymerase reaction. The template should exhibit a strong difference in survival in *ung⁻* versus *ung⁺* hosts. In addition, it should be relatively free of endogenous low-molecular-weight DNA which could prime in vitro DNA synthesis in the absence of added oligonucleotide; such aberrant priming will yield nonmutant clones. A successful in vitro polymerase reac-

tion should convert the oligonucleotide into a fully double-stranded DNA molecule. In the absence of strand displacement of the oligonucleotide (e.g., using T4 DNA polymerase), high efficiency can even be achieved without ligation (Kunkel et al., 1986).

Finally, the oligonucleotide should be of high quality; i.e., purified away from lower molecular weight contaminants that arise from incomplete DNA synthesis. In some cases, especially for oligonucleotides larger than 40 nucleotides, purification by polyacrylamide gel electrophoresis may be necessary (see UNIT 2.7).

Troubleshooting

Incomplete synthesis can result from several factors, including inefficient hybridization of the oligonucleotide primer, inactive (or excess) DNA polymerase, contaminants in the DNA, the polymerase, or the reagents, or a DNA template that contains structures (e.g., hairpin loops) which block polymerization. Such problems must be dealt with on an individual basis, e.g., by varying hybridization conditions, by ensuring the activity of the DNA polymerase, by repurifying the DNA, or by using alternative incubation temperatures or single-stranded DNA binding protein to assist the polymerase in synthesis on unusually difficult templates.

Low biological activity could also result from dNTP contamination by dUTP (e.g., by deamination of dCTP) which, when incorporated in vitro, provides targets for the production of lethal AP sites in the newly synthesized strand containing the desired mutant. For this reason, high-quality dNTP substrates should be used. This eliminates the need for dUTPase pretreatment of the deoxynucleoside triphosphates (dUTPase is not commercially available).

Anticipated Results

With a high-quality template and polymerase reaction, mutants are recovered at 50 to 80%. Thus, direct sequence analysis of random clones to identify the desired mutant(s) is feasible.

Time Considerations

Starting from a single plaque, it should take 1 day to prepare single-stranded template DNA containing uracil residues. Enzyme reactions can be done in 1 day, and reaction products can be introduced into competent *E. coli* cells at the end of that day. Plaques that arise are purified (1 day) and single-stranded DNA ready for DNA sequence analysis is prepared (1 to 2 additional days). DNA sequencing reactions take 1 day, and the results can be seen on the next day. Thus, the entire procedure can take 5 to 7 days. The procedure can be stopped at essentially any step without significant effect on the mutant frequency.

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Key Reference

Kunkel, 1985. See above.

Presents the original description of this technique.

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Mutagenesis with Degenerate Oligonucleotides: Creating Numerous Mutations in a Small DNA Sequence

UNIT 8.2A

BASIC PROTOCOL

A collection of mutagenic oligonucleotides is synthesized by including low concentrations of the three non-wild-type precursors at each step of the synthesis. The frequency of mutation is set by choosing the appropriate amounts of non-wild-type precursors at each step of nucleotide addition. The oligonucleotide also contains an 8-nucleotide palindromic sequence at its 3' end that encompasses a restriction endonuclease site. The palindromic sequence allows nonidentical, but related, mutant DNAs to hybridize at their 3' ends, thereby serving as substrates for DNA polymerase. The resulting molecule is fully double-stranded homoduplex DNA. After digestion with the appropriate restriction endonuclease(s), the double-stranded DNA fragments can be readily cloned to generate a collection of single and multiple point mutants. The protocol is divided into four stages: (1) oligonucleotide design (which includes determination of the mutation frequency, the nucleotides to be mutated, and the structure of the 5' and 3' ends) and subsequent purification after synthesis; (2) mutually primed synthesis to convert the single-stranded oligonucleotide to double-stranded homoduplex form; (3) endonuclease cleavage of the homoduplexes and purification of the final product; and (4) cloning of the double-stranded oligonucleotide into suitable vectors and identification of the mutagenic sequence.

UNIT 8.2B describes related methods for synthesizing long gene segments of any desired sequence.

Materials

- 10× *E. coli* DNA polymerase I buffer (UNIT 3.4)
- 10 mM dNTP mix of 2.5 mM 4dNTP mix (UNIT 3.4)
- [α -³²P]dNTP (400 to 800 Ci/mmol; see UNIT 3.4)
- Klenow fragment of *E. coli* DNA polymerase I (UNIT 3.5)
- 0.5 M EDTA, pH 8.0 (APPENDIX 2)
- TE buffer, pH 7.5 (APPENDIX 2)
- 3 M sodium acetate, pH 5.2
- Buffered phenol (UNIT 2.1)
- Ethanol
- Gel elution buffer: 0.5 M ammonium acetate/1 M EDTA (store protected from light up to several months at room temperature)
- T4 DNA ligase (measured in cohesive-end units; UNIT 3.14)

Additional reagents and equipment for restriction endonuclease digestion (UNIT 3.1), denaturing polyacrylamide gel electrophoresis (UNIT 7.4), phenol extraction and ethanol precipitation (UNIT 2.1), nondenaturing polyacrylamide gel electrophoresis (UNIT 2.7), transformation of *E. coli* (UNIT 1.8), and DNA sequence analysis (Chapter 7)

Design and synthesis of the degenerate oligonucleotide (see Fig. 8.2.1)

1. The oligonucleotide is designed such that the 3' end contains an 8-nucleotide palindromic sequence encompassing a restriction endonuclease cleavage site. The site can be one that gives cohesive or blunt ends.

The palindromic sequence is necessary for hybridization of two oligonucleotides to generate substrates for DNA polymerase. See critical parameters.

2. If possible, the 5' end of the oligonucleotide should consist of sequences encompassing a restriction endonuclease site.

A 5' restriction site is not crucial, since the polymerase reaction will generate blunt ends

Mutagenesis of
Cloned DNA

8.2.1

Contributed by David E. Hill

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Supplement 46

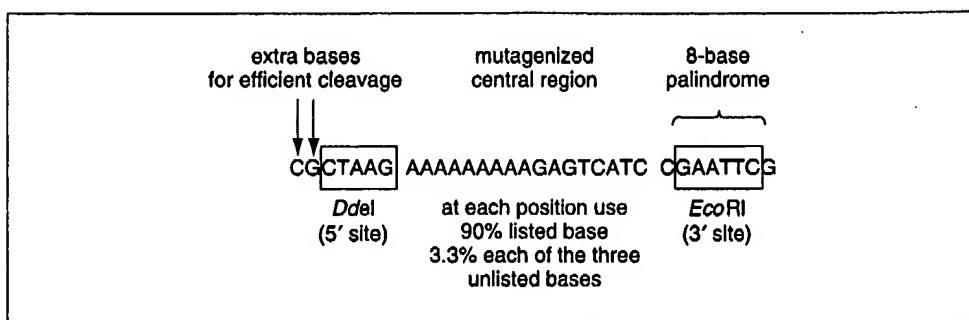


Figure 8.2.1 Design of degenerate oligonucleotide. A 17 base-pair region located between *EcoRI* and *DdeI* sites is mutagenized at a frequency of 10% per position.

suitable for cloning. See critical parameters for further details.

3. The central region contains the mutagenized sequence of interest. Depending on the specific experiment, the length and the mutation frequency of the central region will vary.

The overall limitation on length depends on the efficiency of oligonucleotide synthesis. The phosphite triester method of DNA synthesis as performed by an automated DNA synthesizer can easily produce degenerate oligonucleotides up to at least 100 nucleotides in length.

4. Synthesize the oligonucleotide. At positions where mutations are not desired, such as those comprising the restriction endonuclease cleavage sites, the synthesis is programmed to use homogenous solutions of individual nucleotide precursors. Where mutations are desired, the synthesis is programmed to use defined mixtures of nucleotide precursors.

The frequency of mutation at a given position is determined simply by the relative molarities of the precursors present in the solution. For example, a 10% mutation rate would be achieved with a mixture of 90% wild-type nucleotide and 3.3% each of the three "incorrect" nucleotides. Although newer models of DNA synthesizers are capable of programmed mixing, the most reproducible way to achieve a desired mixture of precursors is to combine appropriate amounts of solid nucleoside phosphoramidites prior to solubilization in anhydrous acetonitrile.

5. Following synthesis, the oligonucleotide is purified by high-performance liquid chromatography and/or electrophoresis in denaturing polyacrylamide gels containing 7 M urea. After purification, the oligonucleotide concentration is adjusted to 1 mg/ml in water (~50 to 100 μ M).

Conversion to double-stranded DNA by mutually primed synthesis (Fig. 8.2.2)

6. Transfer 200 pmol (roughly 1 to 2 μ g) of oligonucleotide to a 500- μ l microcentrifuge tube. Adjust the volume to 7 μ l with water.

The oligonucleotide does not need to be phosphorylated if there will be a restriction site at the 5' end after conversion to double-stranded form. If the 5' end will not contain a restriction site, the oligonucleotide should be phosphorylated by T4 polynucleotide kinase (UNIT 3.10).

7. Incubate the oligonucleotide for 5 min at 70°C; add 1 μ l of 10 \times DNA polymerase I buffer, cool to room temperature, and incubate for at least 60 min.

The second incubation can be done at any temperature that permits hybridization of the 3' end palindromic sequences (typically 23°C).

8. Add 2 μ l of 10 mM 4dNTP mix (2.5 mM each 4dNTP), 5 U Klenow fragment, and 10 μ Ci of any one [α - 32 P]dNTP.

A low level of radioactively labeled dNTP is included in the reaction to facilitate purification of the DNA.



Figure 8.2.2 Mutually primed synthesis for cloning degenerate oligonucleotides. A degenerate oligonucleotide (top line; see Fig. 8.2.1.) is self-annealed via the 8-nucleotide palindrome on the 3' end that encompasses the *EcoRI* site, and then treated with the Klenow fragment. The resulting double-stranded DNA contains two oligonucleotide units each derived from an original oligonucleotide molecule (nucleotides that deviate from the "wild-type" sequence are depicted as large letters). Upon cleavage by *EcoRI* and *DdeI*, the double-stranded oligonucleotides are cloned into an appropriate vector.

9. Incubate 1 hr at 23°C. Add 5 more units of Klenow fragment and continue incubation for 2 hr more (or overnight).
10. Stop the reaction with 1 µl of 0.5 M EDTA, adjust the volume to 50 µl with TE buffer, and add sodium acetate to 0.3 M. Extract once with buffered phenol and precipitate the DNA with ethanol. Resuspend the DNA in 20 µl TE buffer.
11. Remove 2 µl for analysis on denaturing polyacrylamide gels (see step 17).
12. In a 30-µl reaction, digest the now double-stranded oligonucleotide with the restriction endonuclease recognizing the outside sites (originally the 5' site of the single-stranded oligonucleotide).

Because of the high concentration of restriction sites, complete digestion will probably require incubation for at least 2 hr with 10 to 40 U of enzyme per original microgram of oligonucleotide. If the oligonucleotide does not contain a 5' restriction site, cleave the DNA with the enzyme recognizing the internal restriction site (originally at the 3' end of the single-stranded oligonucleotide).

13. After digestion, remove 2 µl for analysis on denaturing polyacrylamide gels (see step 17), then extract the remainder of the reaction mixture with buffered phenol, and concentrate the DNA by ethanol precipitation.
14. Purify the double-stranded oligonucleotide mixture by electrophoresis on non-denaturing polyacrylamide gels. To prevent denaturation of the DNA, do not allow the gel to heat up above room temperature.

This step effectively removes the small DNA fragments generated by the restriction digest as well as unreacted, single-stranded oligonucleotides from the desired product. These

contaminants will reduce the ligation efficiency. In general, it is better to perform the gel purification step after cleavage of the outside restriction sites, but prior to cleavage at the internal site (originally the 3' site of the single-stranded oligonucleotide).

15. After electrophoresis, the desired double-stranded molecule is excised as a band from the gel and eluted in gel elution buffer, as described in UNIT 2.7. Resuspend the double-stranded oligonucleotide mixture in 20 μ l TE buffer and store at -20°C .

Since the double-stranded oligonucleotide is a heterogeneous mixture of different sequences, the DNAs might not migrate as a sharp band, even though they are of identical length. Electroelution of the double-stranded oligonucleotide is an alternate method of extraction (see UNIT 2.6). Be sure that the molecular weight cutoff of the dialysis bag is below that of the oligonucleotide.

16. Digest the double-stranded oligonucleotide with the enzyme recognizing the internal restriction site (the original 3' site) to produce the final product—a double-stranded, homoduplex version of the oligonucleotide mixture with 5' and 3' ends suitable for ligation into standard vectors. Remove 2 μ l for analysis on denaturing polyacrylamide gels (see step 17). Phenol extract the remainder, ethanol precipitate, and resuspend in 20 μ l TE buffer.
17. Analyze the various 2- μ l aliquots (see steps 11, 13, 16) by electrophoresis on denaturing polyacrylamide gels to confirm that the individual reactions have produced the desired products.

Depending on the initial size of the oligonucleotide, 6 to 12% polyacrylamide gels similar to those used for DNA sequencing are employed. ^{32}P end-labeled oligonucleotides of various lengths are useful as size standards.

Cloning of oligonucleotide

18. Serially dilute the double-stranded oligonucleotide in TE buffer by successive factors of 10 until a 10,000-fold dilution is reached.

It is necessary to empirically determine the optimal oligonucleotide concentration for each cloning experiment. However, the empirical test will often generate a sufficient number of colonies that can be subjected to DNA sequence analysis.

19. Set up a series of ligation reactions, each containing a constant amount of vector and a portion of each dilution of oligonucleotide (UNIT 3.16).

The double-stranded oligonucleotides can be ligated directly into either bacteriophage M13-based vectors (UNIT 1.15) or into plasmid vectors. Due to reliable methods of sequencing double-stranded DNA molecules (UNIT 7.4), it is possible to ligate the oligonucleotide into the vector that will be used to determine directly the phenotypes of the mutants.

20. Introduce the ligation mixtures into an appropriate strain of *E. coli* by standard transformation procedures.

In order to avoid DNAs containing multiple oligonucleotide insertions, it is best to analyze colonies that were generated with the lowest amount of oligonucleotide. If the vector was generated by cleavage with two separate restriction enzymes, the background of colonies without inserted oligonucleotides should be low (UNIT 3.16).

21. Analyze the transformants by restriction enzyme digestion and DNA sequence analysis.

If the background is too high, colonies containing inserted oligonucleotides can be identified by colony filter hybridization using 5'- ^{32}P -labeled oligonucleotide as a probe. The procedure for hybridization using oligonucleotide probes is described in UNIT 6.4.

COMMENTARY

Background Information

The procedure described here is an efficient method for using the products of a single oligonucleotide synthesis to create numerous mutations in a small region of DNA (20 to 80 nucleotides). Mutant oligonucleotides are generated during the organic synthesis reaction by including low concentrations of the three non-wild-type nucleotide precursors with the wild-type precursor at each step of the synthesis (see Fig. 8.2.1). The product of such a DNA synthesis is a degenerate oligonucleotide, i.e., a complex mixture of related molecules, each of which has a defined probability of being altered from the wild-type sequence. The frequencies and types of zero, single, double, and higher order mutations can be set simply by choosing the appropriate amounts of non-wild-type precursors at each step of nucleotide addition during the synthesis.

An important feature of the method is that the single-stranded degenerate oligonucleotide is converted to double-stranded homoduplex molecules that can be cloned directly into standard vectors (see Fig. 8.2.2). The palindromic character of the 3' end of the oligonucleotide allows nonidentical DNAs to hybridize; thus, the oligonucleotides serve as mutual primers for extension by the Klenow fragment of *E. coli* DNA polymerase I. The resulting product is a homoduplex of length $2A + 2N + B$ (where A is the length of the 5' flanking sequence, N is the length of the heterogeneous, mutagenized central region, and B is the length of the 3' palindrome flanking region). By cloning homoduplex DNAs, mismatch repair *in vivo* is avoided, potential bias against particular mismatched nucleotides is prevented, and mutations located in all possible positions of a sequence can be obtained.

Since the desired rate of mutation is programmed into the oligonucleotide synthesis and hence is predetermined, the expected results from any particular cloning of degenerate oligonucleotides can be calculated simply using the laws of probability. For example, to maximize the number of single and double point mutants within a 15-bp region of DNA, a 10% mutation rate can be employed. This should give, on average, 1.5 nucleotide substitutions per oligonucleotide. Most of the oligonucleotides will have one or two mutations, while some will have none or more than two. The oligonucleotides lacking any mutations

(i.e., the wild-type sequence) provide a necessary control without requiring an extra synthesis.

For many experiments where it is desired to generate a large number of single base pair substitutions within a given region, low mutation rates (1 to 20% per nucleotide) are typically used. For such experiments, eight mixtures of nucleotide precursors are needed during the synthesis. Four of the mixtures are homogeneous solutions of pure nucleotide precursors such as would be used for conventional oligonucleotides. The remaining four mixtures are heterogeneous solutions composed of one major precursor and predetermined amounts of the three "mutagenic" precursors.

Higher mutation frequencies can be used to saturate a particular site with all possible nucleotide changes. In this case, a separate oligonucleotide is synthesized for each single nucleotide that is to be changed to the three possible non-wild-type nucleotides. At the relevant step in the synthesis, an equimolar mixture of the three non-wild-type nucleotide precursors (i.e., 33% each) replaces the wild-type precursor.

Finally, it is sometimes useful to synthesize "custom-designed" degenerate oligonucleotides. For example, when carrying out a low-frequency mutagenesis as described above, it is extremely difficult to obtain all possible mutations. Indeed, although it is relatively easy to obtain about 70% of the possible changes, continued random sequencing will result in many "repeat" mutations before new ones are obtained. In this case, it is better to design a degenerate oligonucleotide that optimizes the chances of obtaining any "missing" mutations of interest. Many, if not all, of the missing mutations can often be obtained with one or a few additional oligonucleotides.

Literature Review

The use of degenerate oligonucleotides for mutagenesis of small regions of DNA was first described by Matteucci and Heyneker (1983). The protocol described here differs from this and alternative procedures basically in the method of cloning the degenerate oligonucleotides. Previous cloning methods have required the synthesis of complementary DNA strands followed by hybridization. Such hybrids necessarily have one or more mismatches which must be repaired *in vivo* following trans-

formation. The use of mismatch hybrids can reduce overall cloning efficiency and limit the spectrum of single and multiple base changes that occur in a given region of DNA.

The method of mutually primed synthesis described here eliminates cloning bias and positional effects of mismatches because all DNAs are cloned as homoduplex molecules (Hill et al., 1986a,b; Oliphant et al., 1986). In addition, since only a single oligonucleotide synthesis is necessary for obtaining both the wild-type control DNA and a large collection of mutants, significant cost savings can be realized.

Critical Parameters

Since the method relies on efficient organic DNA synthesis, it is essential that the oligonucleotide be synthesized with the freshest possible precursors. This is especially important for the mixed solutions in order to minimize any bias in nucleotide addition. In particular, G residues are sometimes added relatively poorly if the reagents are not fresh. The overall limitation on the length of the region to be mutagenized depends critically on the efficiency of the oligonucleotide synthesis. It is now possible to obtain oligonucleotides that are 100 bases in length, and technological improvements are likely to extend this further. Since 8 to 20 nucleotides at the 5' and 3' ends are constrained by the method, this makes it possible to mutagenize a region as large as 90 bp with a single oligonucleotide. Moreover, larger regions can be mutagenized by using a set of contiguous or overlapping oligonucleotides.

The main critical parameters are the structures at the 5' and 3' ends. The only absolute requirement of the method is that the 3' end be a palindromic sequence that can be cleaved by a restriction endonuclease. The palindrome is necessary for the mutual priming reaction, and the restriction site is necessary for cleavage of the initial product, an oligonucleotide dimer, to oligonucleotide units suitable for cloning. It is preferable if the 5' end also contains a sequence that is recognized by a restriction endonuclease. In the ideal case, cleavage at the 5' site will generate cohesive ends that are different from those generated at the 3' site; this facilitates directional cloning (UNIT 3.16) and makes it possible to perform complex ligation reactions using two different fragments to provide the relevant 5' and 3' joining sites. However, the 5' terminal sequences can be anything; in this

case, the oligonucleotides are cloned via the blunt ends generated by the mutually primed synthesis procedure.

If a restriction site is to be used at both the 5' and 3' end of the oligonucleotide, there is a choice as to which site should be on the different ends. In this case, the restriction site having the highest GC composition should be used at the 3' site in order to facilitate the annealing reaction. For this reason, the palindrome at the 3' end should usually be extended to 8 bases by flanking the site with an additional G or C residue; this has the consequence that the inserted oligonucleotide will contain an extra base between the 3' restriction site and the sequence of interest. Six-base palindromes at the 3' end can suffice for mutually primed synthesis if all residues are GC. However, the reaction is considerably less efficient if other 6-base palindromes are used.

If the 5' end contains a sequence that is to be cleaved with a restriction endonuclease, it is useful to include 1 to 3 extra nucleotides beyond the recognition sequence at the 5' end to facilitate cleavage. In addition, it is advantageous to minimize the length of the palindrome at the 5' end in order to disfavor hybridization that might block complete extension. This can be easily arranged by choosing additional 5'-terminal nucleotides that are not complementary to those immediately flanking the 5' site on the other side.

The choice of vector depends on what is to be done with the mutations once they are obtained. Cloning into standard M13 vectors such as M13mp18 or M13mp19 (UNIT 1.15) has the advantage of easy DNA sequencing; however, the mutated fragments will generally have to be transferred to another molecule for phenotypic analysis. This can be rather cumbersome as it requires preparation of double-stranded M13 DNA (for cloning) in addition to single-stranded DNA (for sequencing). If possible, it is better to clone the degenerate oligonucleotides directly into the molecule that will be used for the phenotypic analysis. By sequencing the double-stranded DNA (UNIT 7.4), only one DNA preparation has to be performed. However, it should be noted that sequencing using the final molecule usually requires a special oligonucleotide primer adjacent to the region of interest.

Anticipated Results

The organic synthesis reaction should easily produce several micrograms of oligonucleo-

tide. After conversion to the double-stranded form, one should have at least 100 ng of DNA. With standard transformation efficiencies, it is possible to generate numerous different mutants and to effectively saturate a small region of DNA with all possible single base changes.

Time Considerations

Typically, oligonucleotides are produced on automated synthesizers that are operated by trained personnel. The actual synthesis and initial purification of the oligonucleotide takes only a few days (waiting for the synthesis facility to make the oligonucleotide can sometimes take a lot longer).

Given the oligonucleotide, conversion to double-stranded form by mutually primed synthesis, the first restriction endonuclease digestion, and nondenaturing gel electrophoresis will require 1 to 2 days. The gel can be run overnight at the end of day 1. Elution of the double-stranded oligonucleotide from the gel, the second restriction digest, and ethanol concentration will require ~1 day. The ligation reaction can be performed overnight at the end of day 2. Transformation and plasmid DNA preparation will require 2 to 3 days. Thus, at the end of 5 days, plasmid or phage DNA can be ready for sequencing to identify the exact

mutation. DNA sequencing will require 1 day with an overnight autoradiographic exposure.

Thus, in 1 week, it is possible to start with a degenerate oligonucleotide and finish with identified mutant DNAs already cloned into vectors suitable for phenotypic analysis.

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Random Mutagenesis by PCR

UNIT 8.3

Error-prone PCR (EP-PCR) is the method of choice for introducing random mutations into a defined segment of DNA that is too long to be chemically synthesized as a degenerate sequence. For shorter (<100 bp) DNA segments, the most powerful method is to chemically synthesize the sequence with a low degree of nucleotide degeneracy (UNIT 8.2A). This method, however, is too expensive and time-consuming to be used for mutagenizing long stretches of DNA. Using EP-PCR, the 5' and 3' boundaries of the mutated region may be defined by the choice of PCR primers. Accordingly, it is possible to mutagenize an entire gene or merely a segment of a gene. The average number of mutations per DNA fragment can be controlled as a function of the number of EP-PCR doublings performed.

The EP-PCR technique described here is based on the protocol of Cadwell and Joyce (1992). The Basic Protocol is for a 400-bp sequence; the Alternate Protocol is for a library. EP-PCR takes advantage of the inherently low fidelity of *Taq* DNA polymerase, which may be further decreased by the addition of Mn^{2+} , increasing the Mg^{2+} concentration, and using unequal dNTP concentrations.

STRATEGIC PLANNING

After choosing a region of DNA to randomly mutagenize, one must decide on the desired level of mutagenesis that is best suited to the project. If the mutation rate is too low, it may not be possible to find the potentially rare variants of interest. If the mutation rate is too high, nearly all of the resulting library molecules will carry multiple mutations and may therefore be inactive. The desired extent of mutation depends on the type of activity one is attempting to generate and the number of library members that can be screened. For example, if one is attempting to generate an activity that may require multiple mutations, such as a novel binding or catalytic activity, then it may be necessary to generate molecules with several mutations per template. In this case it would also be important to have a screening or selection protocol that would allow for the evaluation of a large number of variants. On the other hand, if one is looking for mutations causing loss of protein function or decrease in thermal stability, for example, then 1 to 2 mutations per template may be ideal. In this case it may be sufficient to screen a smaller number of

Table 8.3.1 Average Number of Mutations per DNA Template as a Function of Template Length and Number of EP-PCR Doublings

EP-PCR doublings	Mutations per nucleotide position	Template length				
		100 bp	200 bp	400 bp	800 bp	1600 bp
5	0.0033	0.33	0.66	1.3	2.6	5.3
10	0.0066	0.66	1.3	2.6	5.3	11
20	0.013	1.3	2.6	5.3	11	21
30	0.020	2.0	4.0	7.9	16	32
50	0.033	3.3	6.6	13	26	53

variants. A reasonable approach in many instances is to generate a library such that a few unmutagenized molecules will be present in the collection of screened clones.

The average number of mutations per template increases as a function of the number of doublings in the EP-PCR reaction (Table 8.3.1). It should be noted that it is the number of doublings that is the determining factor, rather than the number of EP-PCR cycles. Each cycle of EP-PCR generally increases the amount of DNA by a factor of 1.7 to 1.9 until the DNA concentration reaches a plateau and then stops increasing altogether. The point at which this plateau occurs depends on the template and primer lengths and sequences, but is generally in the range of 5 to 50 ng/μl. It is not advisable to continue thermal cycles beyond the plateau point.

Prior to performing the actual EP-PCR reaction, it is important to run a pilot reaction to determine the amplification efficiency under the EP-PCR reaction conditions. This should be evaluated for two reasons. First, if the amplification per cycle is too low (<1.7-fold increase in product DNA concentration per cycle), DNA fragments that contain one or both of the primer-binding sites, but are shorter than the desired product, may have a strong selective advantage for amplification. These shorter fragments are produced by mispriming during normal or error-prone PCR. After several cycles, these shorter sequences may "take over" the EP-PCR reaction. This can be an especially severe problem when many cycles (>15) are to be performed. Second, the amplification per cycle must be known in order to calculate the number of EP-PCR cycles necessary to achieve the desired number of doublings.

The amount of DNA amplification per EP-PCR cycle can be determined by diluting a known amount of the unmutagenized PCR product, then amplifying it using the EP-PCR protocol and occasionally removing portions of the reaction for quantitation on an ethidium bromide-stained agarose gel (UNIT 2.7). The amplification per cycle should generally be >1.7. The yield per cycle can be optimized by altering the annealing temperature. Also, the extension time should last for at least 3 min, to ensure complete extension. If this does not bring satisfactory results, longer or shorter primers may have to be used. Primer lengths of 20 to 40 nucleotides usually produce acceptable results. Other parameters may also be optimized to improve the amplification, as described in UNIT 15.1 (the Mg²⁺ concentration should not be reduced, however, as this may lead to increased fidelity of DNA synthesis).

Table 8.3.2 Fraction of Nonmutated DNA Templates as a Function of Template Length and Number of EP-PCR Doublings

EP-PCR doublings	Mutations per nucleotide position	Template length				
		100 bp	200 bp	400 bp	800 bp	1600 bp
5	0.0033	0.72	0.52	0.27	0.071	0.0050
10	0.0066	0.52	0.27	0.071	0.0050	2.5×10^{-5}
20	0.013	0.26	0.070	0.0049	2.4×10^{-5}	5.8×10^{-10}
30	0.020	0.14	0.018	0.00033	1.1×10^{-7}	1.3×10^{-14}
50	0.033	0.035	0.0012	1.5×10^{-6}	2.2×10^{-12}	4.8×10^{-24}

Table 8.3.1 outlines the average number of nucleotide substitutions per template as a function of the number of EP-PCR doublings and the length of the template. Table 8.3.2 shows what fraction of the resulting products will be completely free from mutation.

In most cases, the mutagenized DNA of interest will encode a protein. The fraction of mutated amino acids will be higher than the fraction of mutated nucleotides by a factor of ~2.2. This is because a mutation in any of the three positions of a codon may result in an amino acid substitution. If the initial template is a random open reading frame (i.e., equal probability of each nucleotide at each position in each codon), mutation at the first position of a codon will cause an amino acid change 96% of the time; mutation at the second and third positions will cause amino acid changes 100% and 23% of the time, respectively (as calculated using the mutation frequencies in Table 8.3.6; see Anticipated Results).

MUTAGENESIS OF A DNA SEQUENCE

In this protocol, a 400-bp DNA sequence is mutagenized for ten doublings, to achieve a mutation rate of 0.66% per nucleotide position.

Materials

- 100 mM Tris-Cl, pH 8.3 (APPENDIX 2)
- 2 M KCl
- 200 mM MgCl₂
- 25 mM dCTP, pH ~7
- 25 mM dTTP, pH ~7
- 5 mM dATP, pH ~7
- 5 mM dGTP, pH ~7
- 100 μM each 5' and 3' PCR primers
- 200 pg/μl DNA template (400 bp in length)
- 25 mM MnCl₂
- 5 U/μl *Taq* DNA polymerase
- 100-μl PCR tubes (Sarstedt)
- Thermal cycler (see UNIT 15.1)
- TOPO T/A cloning kit (Invitrogen)
- QIAprep kit (Qiagen)
- Additional reagents and equipment for PCR amplification (UNIT 15.1) and agarose gel electrophoresis (UNIT 2.7)

1. Make up the following PCR reaction mixture in a 100-μl PCR tube on ice:

- 51 μl water
- 10 μl 100 mM Tris-Cl, pH 8.3 (10 mM final)
- 2.5 μl 2 M KCl (50 mM final)
- 3.5 μl 200 mM MgCl₂ (7 mM final)
- 4 μl 25 mM dCTP (1 mM final)
- 4 μl 25 mM dTTP (1 mM final)
- 4 μl 5 mM dATP (0.2 mM final)
- 4 μl 5 mM dGTP (0.2 mM final)
- 2 μl 100 μM 5' primer (2 μM final)
- 2 μl 100 μM 3' primer (2 μM final)
- 10 μl 200 pg/μl template DNA (20 pg/μl final)
- 2 μl 25 mM MnCl₂ (0.5 mM final)
- 1 μl 5 U/μl *Taq* DNA polymerase (0.05 U/μl final)
- Total volume, 100 μl.

BASIC PROTOCOL

Mutagenesis of Cloned DNA

8.3.3

The $MnCl_2$ should be added immediately before the thermal cycling is initiated. The Taq DNA polymerase should be added when the thermal cycling reaction has reached the first annealing step.

2. Place the tube in the thermal cycler and perform ~12 PCR cycles (UNIT 15.1), or enough to obtain a 1000-fold (10 doublings) increase in the amount of PCR product relative to the input template.

The cycling conditions will vary depending on the template and primers, but reasonable starting conditions are: 94°C for 1 min (denaturation), 60°C for 1 min (annealing), and 72°C for 3 min (extension).

The annealing temperature should be kept >50°C, if possible, to avoid mispriming, the frequency of which increases at the high divalent cation concentration used for EP-PCR. The 3-min extension time reduces the selective amplification of shorter, undesirable sequences produced by mispriming.

3. Run an ethidium bromide-containing agarose gel to confirm the amount and correct molecular weight of the product (UNIT 2.7).
4. Clone and sequence a sample of the resulting PCR DNA to determine the frequency of mutations in the product using the TOPO T/A cloning kit and the QIAprep kit.

For information on DNA sequencing, see Chapter 7.

To achieve higher levels of mutagenesis, the template in the initial reaction will need to be diluted to a greater extent, and more EP-PCR cycles will need to be performed. Also, if more than ~15 cycles of EP-PCR are to be performed, a fresh aliquot of Taq polymerase should be added after the 15th cycle.

One problem that often occurs when attempting to achieve a large number of doublings is that PCR products that are smaller than the desired one "take over" the PCR reaction. If this happens, one should first make sure that the EP-PCR conditions are optimized, resulting in an increase in DNA product of at least 1.7-fold per cycle. This may require increasing the extension time to over 3 min, especially when the desired product is >1 kb. Another way to avoid conditions that selectively amplify shorter templates is to increase the denaturing time (up to 75 sec). Also, the annealing temperature should be as high as possible to minimize the occurrence of mispriming events. The highest annealing temperature that gives efficient amplification must be determined empirically. UNIT 15.1 describes other parameters that can be varied to optimize the amplification reaction. If these measures still fail to eliminate the problem, it may be necessary to perform a smaller number of cycles (using a higher starting template concentration), and then gel purify the full-length PCR product before continuing with more thermal cycling. In some cases, it may be necessary to perform this gel purification step periodically (e.g., every 8 cycles). One can use agarose gel purification (UNIT 2.7) which is very easy, sensitive, and convenient. However, polyacrylamide gel electrophoresis (PAGE; UNIT 2.7) can accomplish a higher degree of purification.

MUTAGENIZING A LIBRARY OF SEQUENCES

Sometimes it is desirable to mutagenize an entire collection of sequences simultaneously. The Basic Protocol is appropriate in cases where the starting template is a unique sequence, but the following modifications are recommended when the starting template is itself a library.

The Basic Protocol calls for the EP-PCR reaction to be initiated with a very small amount of template, but this amount may be insufficient to preserve the initial library complexity. To avoid complexity loss before and during the amplification process, one can start with a comparatively large template concentration and perform only four EP-PCR cycles, and then transfer ~10% of the resulting material into a fresh EP-PCR reaction. These "serial transfers" are continued until the desired number of doublings is achieved. One additional advantage of this method is that the progress of the EP-PCR reaction can be monitored throughout the entire procedure, a luxury that is not possible using the Basic Protocol.

This protocol will give ~50 EP-PCR doublings and results in mutations in ~3.5% of the nucleotide positions in the DNA template. However, the actual mutagenic rate may vary with conditions and template.

Additional Materials (also see Basic Protocol)

30 ng/μl DNA template (library)

1. Make up the following EP-PCR reaction mixture on ice:

960 μl water
150 μl 100 mM Tris-Cl, pH 8.3 (10 mM final)
37.5 μl 2 M KCl (50 mM final)
52.5 μl 200 mM MgCl₂ (7 mM final)
60 μl 25 mM dCTP (1 mM final)
60 μl 25 mM dTTP (1 mM final)
60 μl 5 mM dATP (0.2 mM final)
60 μl 5 mM dGTP (0.2 mM final)
30 μl 100 μM 5' primer (2 μM final)
30 μl 100 μM 3' primer (2 μM final)
Total volume, 1500 μl.

2. Divide the EP-PCR reaction mixture into 16 labeled tubes suitable for 100 μl PCR reactions (i.e., into 90-μl aliquots).

These may be stored at 4°C for a few hours.

3. Add 7 μl of the 30 ng/μl DNA library to tube 1 to give ~2 ng/μl. Place tube 1 in the thermal cycler; once it has reached the annealing temperature, add the following and mix:

2 μl 25 mM MnCl₂ (0.5 mM final)
1 μl 5 U/μl *Taq* DNA polymerase (0.05 U/μl final).

ALTERNATE PROTOCOL

4. Perform four cycles of EP-PCR amplification (see Basic Protocol, step 2 annotations, for discussion of conditions). During the final extension at 72°C, place tube 2, containing the fresh EP-PCR mixture into the same PCR block. Before the final extension is completed on tube 1, but ensuring that tube 2 has reached the extension temperature, transfer 10 µl of EP-PCR reaction mixture from tube 1 into tube 2, and then add the following to tube 2 and mix:

2 µl 25 mM MnCl₂ (0.5 mM final)
1 µl 5 U/µl *Taq* DNA polymerase (0.05 U/µl final).

Remove tube 1 from the block and store at 4°C.

The numbers given here for starting DNA template concentration and transfer volume may need to be modified in accordance with results from pilot EP-PCR reactions, which serve to determine the amplification efficiency.

5. Repeat step 4 for the remaining 14 tubes. Analyze the PCR reaction using agarose gel electrophoresis (UNIT 2.7) after every fourth transfer, and quantitate the bands in successive PCR amplifications.

The DNA amplification per EP-PCR cycle should not decrease to below 1.7, even for the fourth cycle. It is also important that the amount of DNA at the end of the four EP-PCR cycles not increase from transfer to transfer. If this does occur, reduce the transfer volume.

Before the entire EP-PCR protocol is attempted, it is important to pilot the EP-PCR conditions to ensure that low-molecular-weight PCR products are not "taking over" the reaction, and that the amplification per cycle is at least 1.7. The optimal PCR amplification conditions may be different from normal PCR amplification performed upon the same library.

This serial transfer approach yields a succession of samples with increasing levels of mutagenesis. If one is uncertain about the optimal level of mutagenesis for a particular application, the samples from different stages of the EP-PCR procedure can be mixed prior to screening or selection.

COMMENTARY

Background Information

The simplest and most versatile method for introducing random point mutations into a DNA sequence is to take advantage of the inherently low fidelity of *Taq* DNA polymerase. This enzyme can misincorporate with a frequency as high as 0.02% per position (Eckert and Kunkel, 1991). By performing exponential amplification through PCR, these errors will accumulate. Leung et al. (1989) showed that the fidelity of copying by *Taq* polymerase can be further decreased by skewing the relative dNTP concentrations, using a high Mg²⁺ concentration, and including Mn²⁺ in the reaction mixture. Cadwell and Joyce (1992) used a similar strategy to obtain an error rate per nucleotide position of about 0.066% per PCR cycle. This protocol, which forms the basis of that described here, has many useful qualities: (1) it is

extremely simple in that it does not require any unusual reagents such as nucleotide analogs; (2) the EP-PCR efficiency is comparable to that for standard PCR conditions; (3) in many instances the desired level of mutagenesis can be obtained in ~1 hr with a single PCR reaction; and (4) a wide spectrum of nucleotide substitutions is generated. Alternative, more complex procedures also exist that utilize multiple parallel EP-PCR reaction conditions with different dNTP ratios, or use nucleotide analogs (Spee et al., 1993; Fromant et al., 1995; Xu et al., 1999). Vartanian et al. (1996) have described a protocol that can result in very high mutagenic levels.

Critical Parameters

The key to success in this technique is to characterize the amplification efficiency of the

Table 8.2.3 Troubleshooting Guide for Random Mutagenesis by PCR

Problem	Possible cause	Solution
No EP-PCR product	EP-PCR conditions need optimizing	Optimize pilot EP-PCR reaction, especially with regard to annealing temperature. Use long extension times (at least 3 min). If this fails, test primers, template, and other reagents under "normal" PCR conditions to ensure that there has not been a primer design or synthesis error, or a degeneration in reagent quality.
Multiple EP-PCR products of incorrect lengths	EP-PCR conditions need optimizing	Optimize pilot EP-PCR reaction, as above. If this fails to solve the problem, periodically gel purify the product of correct length.
Brown precipitate observed in EP-PCR reaction mixture	Manganese salts are precipitating out of solution	Add manganese to EP-PCR reaction mixture just prior to thermal cycling.
Successive transfers contain decreasing amounts of DNA when visualized on an agarose gel	Transfer volume is too small	Increase transfer volume
Successive transfers contain increasing amounts of DNA when visualized on an agarose gel	Transfer volume is too large, possibly because the efficiency of the PCR reaction is increasing as the most easily amplified sequences begin to dominate the mixture	Decrease transfer volume

EP-PCR reaction and confirm that the DNA concentration is increasing by at least 1.7-fold per cycle. Once the amplification efficiency is known, it will be easy to calculate the number of EP-PCR cycles that must be performed in order to achieve the desired level of mutagenesis. These issues are discussed in detail above (see Strategic Planning).

Troubleshooting

Guidelines for troubleshooting experimental difficulties are presented in Table 8.2.3.

Anticipated Results

The degree of mutagenesis achieved is a function of the number of EP-PCR doublings performed, and therefore can be controlled by the investigator. Anticipated levels of mutagenesis at the DNA, and when applicable, the protein level, are described in Tables 8.3.1, 8.3.2, 8.3.3, 8.3.4, and 8.3.5, and discussed above in Strategic Planning. It is important,

however, to verify that the intended degree of mutagenesis has been achieved by sequencing a few of the resulting, cloned DNA molecules.

The EP-PCR protocol produces all types of substitution mutations, but the distribution is highly nonrandom. The results obtained by the authors in one experiment are shown in Table 8.3.6. It should be noted that in the EP-PCR reaction, both top and bottom DNA strands are equally subject to mutagenesis, so mutations from G to A and from C to T, for example, cannot be distinguished from each other; consequently these are combined together in the table.

Time Considerations

The amount of time required to determine the amplification efficiency of the EP-PCR is 3 hr. If necessary, optimizing the conditions to improve the EP-PCR efficiency requires 1 day. Performing the actual EP-PCR and verifying the total number of doublings achieved takes

Mutagenesis of
Cloned DNA

8.3.7

Table 8.3.4 Average Number of Amino Acid Mutations per Open Reading Frame (ORF) as a Function of ORF Length and Number of EP-PCR Doublings

EP-PCR doublings	Mutations per codon	ORF length				
		100 bp	200 bp	400 bp	800 bp	1600 bp
5	0.0076	0.25	0.50	1.0	2.0	4.0
10	0.015	0.50	1.0	2.0	4.0	8.0
20	0.030	1.0	2.0	4.0	8.1	16
30	0.045	1.5	3.0	6.0	12	24
50	0.076	2.5	5.0	10	20	40

Table 8.3.5 Fraction of ORF's Encoding Wild-Type Polypeptide as a Function of ORF Length and the Number of EP-PCR Doublings

EP-PCR doublings	Mutations per codon	ORF length				
		100 bp	200 bp	400 bp	800 bp	1600 bp
5	0.0076	0.78	0.60	0.36	0.13	0.017
10	0.015	0.60	0.36	0.13	0.017	0.00030
20	0.030	0.36	0.13	0.017	0.00028	7.8×10^{-8}
30	0.045	0.21	0.045	0.0021	4.3×10^{-6}	1.8×10^{-11}
50	0.076	0.073	0.0053	2.8×10^{-5}	7.9×10^{-10}	6.3×10^{-19}

Table 8.3.6 Observation of Each Type of Substitution in a Collection of 97 Mutations Generated by EP-PCR

Type of mutation	Number times observed
A→T and T→A	34
G→A and C→T	26
A→G and T→C	24
A→C and T→G	6
G→C and C→G	5
G→T and C→A	2

anywhere between 2 hr and 1 day, depending on the number of EP-PCR cycles performed. It may take one extra day if periodic gel purification of full-length EP-PCR product must be performed. Cloning a sample of the EP-PCR product may be carried out in 1 day. DNA minipreps for sequencing may be prepared in 1 day. Sequencing the DNA to determine the level of mutagenesis may require 1 to 5 days, depending on the sequencing facility. Analyzing the sequencing results and calculating the degree of mutagenesis takes 1 day. If the level of mutagenesis is significantly different from what was desired, one may have to repeat the EP-PCR, then resequence and determine the level of mutagenesis once again.

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QUANTITATION OF PROTEINS

SECTION I

The success or failure of protein-centered projects can frequently be traced to the quality of the analytical procedures used to characterize the sample at different stages. Qualitative and quantitative analysis can aid in definition of the sample for the purpose of designing separations. A knowledge of the properties of the desired protein (e.g., whether it has a high aromatic amino acid content) can suggest methods of analysis that will help locate the desired protein in a complex mixture. Establishing the properties of an isolated protein creates benchmarks that future researchers can use to evaluate their protocols and final product. Accurate quantitation of the amount of protein at the beginning, middle, and end of a series of steps is the only valid way to evaluate the overall yield of a procedure. The observation of significant loss of protein, without a substantial increase in the purity of the desired protein, following a particular purification procedure would indicate that the procedure should be omitted or revised.

Several spectroscopic procedures for characterizing protein samples are described in *UNIT 10.1A*. Measuring the absorbance of the aromatic amino acids in a protein at difference wavelengths yields a very useful measure of protein concentration. This is non-destructive and requires very little sample or time. A more qualitative, but much more sensitive, evaluation is provided by fluorescence spectroscopy. Quantitation of the amount of protein contained in a solution also can be conveniently accomplished using colorimetric methods. The Bradford and the Lowry methods are the most frequently used and reliable procedures. The method of choice is the Bradford method, which is easy and rapid to complete.

Another approach is amino acid analysis—qualitative analysis to determine purity and quantitative analysis to provide concentrations—both of which are presented in *UNIT 10.1B*. Procedures are also given for calculating amino acid composition from primary analytical data. Significant advances that have improved the precision and sensitivity of amino acid analysis have reinvigorated this method, which had for some years been neglected.

Spectrophotometric and Colorimetric Determination of Protein Concentration

This unit describes spectrophotometric and colorimetric methods for measuring the concentration of a sample protein in solution. In Basic Protocol 1, absorbance measured at 280 nm (A_{280}) is used to calculate protein concentration by comparison with a standard curve or published absorptivity values for that protein (a_{280}). In Alternate Protocol 1, absorbance measured at 205 nm (A_{205}) is used to calculate the protein concentration. The A_{280} and A_{205} methods can be used to quantitate total protein in crude lysates and purified or partially purified protein. Both of these methods are simple and can be completed quickly. The A_{280} method is the most commonly used. The A_{205} method can detect lower concentrations of protein and is useful for dilute protein samples, but is more susceptible to interference by reagents in the protein sample than the A_{280} method. Basic Protocol 2 uses a spectrofluorometer or a filter fluorometer to measure the intrinsic fluorescence emission of a sample solution; this value is compared with the emissions from standard solutions to determine the sample concentration. The fluorescence emission method is used to quantitate purified protein. This simple method is useful for dilute protein samples and can be completed in a short amount of time. The Bradford colorimetric method, based upon binding of the dye Coomassie brilliant blue to an unknown protein, is presented in Basic Protocol 3; the Lowry method, which measures colorimetric reaction of tyrosyl residues in an unknown, is given in Alternate Protocol 2.

BASIC PROTOCOL 1

USING A_{280} TO DETERMINE PROTEIN CONCENTRATION

Determination of protein concentration by measuring absorbance at 280 nm (A_{280}) is based on the absorbance of UV light by aromatic amino acids in protein solutions—due primarily to tryptophan and tyrosine residues and to a lesser extent phenylalanine residues. The measured absorbance of a protein sample solution is used to calculate the concentration either from its published absorptivity at 280 nm (a_{280}) or by comparison with a calibration curve prepared from measurements with standard protein solutions. This assay can be used to quantitate solutions with protein concentrations of 20 to 3000 $\mu\text{g/ml}$.

Materials

- 3 mg/ml spectrophotometric standard protein solution (see recipe; optional)
- Sample protein
- Spectrophotometer with UV lamp

1. For calibrating with standards, use the 3 mg/ml standard protein solution to prepare dilutions of 20, 50, 100, 250, 500, 1000, 2000, and 3000 $\mu\text{g/ml}$ in the same solvent as used to prepare the sample protein. Prepare a blank consisting of solvent alone.

Ideally, for purified or partially purified protein, the protein standard should have an aromatic amino acid content similar to that of the sample protein. For the total protein of a crude lysate, bovine serum albumin (BSA) is a commonly used standard for spectrophotometric quantitation of protein concentration. A 3 mg/ml solution of BSA should have an A_{280} of 1.98, based on an A_{280} of 6.61 for a 1% (w/v) solution.

2. Turn on the UV lamp of the spectrophotometer and set the wavelength to 280 nm. Allow the instrument to warm up 30 min before taking measurements.
3. Zero the spectrophotometer with the solvent blank.

4. Measure the absorbance of the protein standard and unknown solutions.

If the A_{280} of the sample protein is >2.0 , dilute the sample further in the same solvent and measure the A_{280} again.

- 5a. *If the a_{280} of the protein is known:* Calculate the unknown sample concentration from its absorbance value using the following equation, where a_{280} has units of ml/mg cm and b is the path length in cm.

$$\text{concentration (mg/ml)} = \frac{A_{280}}{a_{280} \times b}$$

- 5b. *If standard solutions are used for quantitation:* Create a calibration curve by either plotting or performing regression analysis of the A_{280} versus concentration of the standards. Use the absorbance of the sample protein to determine the concentration from the calibration curve.

USING A_{205} TO DETERMINE PROTEIN CONCENTRATION

Determination of protein concentration by measurement of absorbance at 205 nm (A_{205}) is based on absorbance by the peptide bond. The concentration of a protein sample is determined from the measured absorbance and the absorptivity at 205 nm (a_{205}). This assay can be used to quantitate protein solutions with concentrations of 1 to 100 $\mu\text{g/ml}$ protein.

Additional Materials (also see Basic Protocol 1)

Brij 35 solution: 0.01% (v/v) Brij 35 (Sigma) in an aqueous solution appropriate for dissolving or diluting the sample protein

1. Dissolve or dilute the protein sample in Brij 35 solution.
 2. Turn on the UV lamp of the spectrophotometer and set the wavelength to 205 nm. Allow the instrument to warm up 30 min before taking measurements.
 3. Zero the spectrophotometer with the Brij 35 solution alone.
 4. Measure the absorbance of the sample protein.
- 5a. *If the a_{205} of the protein is known:* Use the equation relating A_{280} and protein concentration (see Basic Protocol 1, step 5a) to calculate the concentration of the sample protein, *except* substitute the appropriate values for A_{205} and a_{205} .
 - 5b. *If the a_{205} is not known:* Estimate the concentration of the sample protein from its measured absorbance using the following equation, where the absorptivity value, 31, has units of ml/mg cm and b is the path length in cm.

$$\text{concentration (mg/ml)} = \frac{A_{205}}{31 \times b}$$

The absorptivity value of 31 ml/mg cm is an average derived from measurement of ten purified proteins (Scopes, 1974). The proteins were first dried and then several dilutions were made in buffer; the a_{205} for each protein was calculated from the absorbance readings of the dilutions.

ALTERNATE PROTOCOL 1

USING FLUORESCENCE EMISSION TO DETERMINE PROTEIN CONCENTRATION

Protein concentration can also be determined by measuring the intrinsic fluorescence based on fluorescence emission by the aromatic amino acids tryptophan, tyrosine, and/or phenylalanine. Usually tryptophan fluorescence is measured. The fluorescence intensity of the protein sample solution is measured, and the concentration of the protein sample solution calculated from a calibration curve based on the fluorescence emission of standard solutions prepared from the purified protein. This assay can be used to quantitate protein solutions with concentrations of 5 to 50 $\mu\text{g/ml}$.

Materials

Spectrophotometric protein standard solution (see recipe) prepared using the purified protein

Sample protein

Spectrofluorometer *or* filter fluorometer with an excitation cutoff filter ≤ 285 nm and an emission filter > 320 nm

1. Prepare dilutions of the purified protein at 5, 7.5, 10, 25, and 50 $\mu\text{g/ml}$ in the same solvent as the sample protein. Prepare a blank consisting of solvent alone.
2. Turn on the lamp of the instrument and allow it to warm up 30 min before taking measurements.

If a spectrofluorometer is used, set the excitation wavelength to 280 nm and the emission wavelength to between 320 and 350 nm. If the exact emission wavelength is not known, determine it empirically by scanning the standard solution with the excitation wavelength set to 280 nm. If the instrument is a filter fluorometer, use an excitation cutoff filter ≤ 285 nm and an emission filter > 320 nm.

3. Zero the instrument with the solvent blank.
4. Measure the fluorescence of the protein standard and sample protein solutions.
5. Create a calibration curve by either plotting or performing regression analysis of the fluorescence intensity versus concentration of the standards. Using the fluorescence intensity of the sample protein, determine the concentration from the calibration curve.

Fluorescence emission is a linear function of concentration only over a limited range.

USING THE BRADFORD METHOD TO DETERMINE PROTEIN CONCENTRATION

The Bradford method depends on quantitating the binding of a dye, Coomassie brilliant blue, to an unknown protein and comparing this binding to that of different amounts of a standard protein, usually BSA. It is designed to quantify 1 to 10 μg protein. Protein determinations in the range of 10 to 100 μg may be carried out by increasing the volume of the dye solution 5-fold and using larger tubes.

Materials

Colorimetric standard protein solution (0.5 mg/ml BSA; see recipe)

0.15 M NaCl

Coomassie brilliant blue solution (see recipe)

1 ml (1-cm-path-length microcuvette)

1. Into eight microcentrifuge tubes place duplicate aliquots of 0.5 mg/ml BSA (5, 10, 15, and 20 μl) and dilute each to 100 μl with 0.15 M NaCl. Into two more microcentrifuge tubes, place 100 μl each of 0.15 M NaCl; these are blank tubes.

2. Add 1 ml Coomassie brilliant blue solution and vortex. Allow to stand 2 min at room temperature.
3. Determine the A_{595} using a 1-cm-path-length (1 ml) microcuvette and make a standard curve by plotting absorbance at 595 nm versus protein concentration. Determine the absorbance for the unknown and use the standard curve to determine the concentration of protein in the unknown.

If the unknown protein concentration is too high, dilute the protein, assay a smaller aliquot of the unknown, or generate another standard curve in a higher concentration range (e.g., 10 to 100 μ g).

USING THE LOWRY METHOD TO DETERMINE PROTEIN CONCENTRATION

The Lowry method depends on quantitating the color obtained from the reaction of Folin-Ciocalteu phenol reagent with the tyrosyl residues of an unknown protein and comparing this color value to the color values derived from a standard curve of a standard protein, usually BSA. This assay is designed to quantify 1 to 20 μ g protein. Protein determinations in the range of 5 to 100 μ g may be carried out by increasing all the volumes 5-fold.

Additional Materials (also see Basic Protocol 3)

- 0.15% (w/v) sodium deoxycholate
- 72% (w/v) trichloroacetic acid (TCA)
- Copper tartrate/carbonate (CTC) solution (see recipe)
- 20% (v/v) Folin-Ciocalteu reagent

1. Into eight microcentrifuge tubes place duplicate aliquots of 0.5 mg/ml BSA (10, 20, 30, and 40 μ l) and dilute each to 200 μ l with water. Into two more microcentrifuge tubes, place 200 μ l water; these are blank tubes.
2. Add 20 μ l of 0.15% sodium deoxycholate to each tube and vortex. Allow to stand 10 min at room temperature.
3. Add 20 μ l of 72% TCA to each tube and vortex.
4. Microcentrifuge 15 min at 3000 \times g, then decant supernatant (protein will be in the precipitate).
5. Dissolve protein pellets in 200 μ l water. Add 200 μ l water to the blank tubes.
6. Add 200 μ l CTC solution, vortex, and allow to stand 10 min at room temperature.
7. Add 100 μ l of 20% Folin-Ciocalteu reagent, *immediately* vortex, and allow to stand 30 min at room temperature.
8. Determine the A_{750} and compare against standard curve (see Basic Protocol 3, step 3).

ALTERNATE PROTOCOL 2

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

Colorimetric standard protein solution (0.5 mg/ml BSA)

Prepare a 10 mg/ml solution of BSA and measure its A_{280} in a 1-cm-path-length microcuvette. The A_{280} should equal 6.61 (i.e., a 0.5 mg/ml solution will have an $A_{280} = 0.33$).

Coomassie brilliant blue solution

In a 1-liter volumetric flask, dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol. Add 100 ml of 85% phosphoric acid. Bring to volume with water. Filter through Whatman no. 1 filter paper. Store at 4°C.

Commercial kits are available from Pierce and Bio-Rad.

Copper tartrate/carbonate (CTC) solution

CTC stock solution: To 50 ml of a solution of 0.2% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.2% (w/v) potassium tartrate, slowly add, while vigorously stirring, 50 ml of a solution of 20% sodium carbonate. This solution is stable >8 weeks at room temperature.

CTC working solution: Mix equal volumes of CTC, 0.8 M NaOH solution, 10% (w/v) SDS solution, and water. The reagent is stable 1 to 2 weeks at room temperature.

Spectrophotometric standard protein solution, 3 mg/ml

Weigh out dry protein and prepare a stock solution at a concentration of 3 mg/ml in the same solvent as used for the sample protein. Store up to 3 months at -20°C.

To prepare calibration standard solutions, dilute the stock solution in solvent to give the desired final concentrations for the standard curve.

Bovine serum albumin (BSA, fraction V; Sigma) is frequently used as a protein standard solution. A 3 mg/ml solution of BSA should have an A_{280} of 1.98, based on an A_{280} of 6.61 for a 1% (w/v) solution.

For quantitation of a purified or partially purified protein, the protein standard should, if possible, have an aromatic amino acid content similar to that of the sample protein.

COMMENTARY

Background Information

Measuring absorbance at 280 nm (A_{280}) is one of the oldest methods for determining protein concentration (Warburg and Christian, 1942; Layne, 1957). This method is still widely used, because it is simple and does not require incubating the sample with exogenous chromophores. However, the detection limit is higher than with colorimetric methods and therefore higher concentrations of protein are necessary. The A_{280} method requires that the protein being quantitated contain aromatic amino acids, primarily tryptophan and tyrosine. Because of the variability in aromatic amino acid content among different proteins, their absorptivity at 280 nm (a_{280}) also varies. Therefore, if calibration standards are used for quantitation, the aromatic amino acid content of the standard must be similar to that of the sample protein for

accurate results. Accordingly, the quantitation of proteins by peptide bond absorption at 205 nm (A_{205}) is more universally applicable. Furthermore, the absorptivity for a given protein at 205 nm is several-fold greater than that at 280 nm (Scopes, 1974; Stoscheck, 1990). Thus, lower concentrations of protein can be quantitated with the A_{205} method. The disadvantage of this method is that some buffers and other components absorb at 205 nm (Stoscheck, 1990).

In addition to the aromatic amino acids, several others have absorption maxima in the UV range. Table 10.1A.1 shows the wavelengths of absorption maxima and corresponding molar absorptivity (ϵ) for the amino acids with appreciable absorbance in the UV range. Only tryptophan has an absorption maximum near 280 nm, although tyrosine will absorb

Table 10.1A.1 Absorption Maxima and Molar Absorptivity (ϵ) of Amino Acids^a

Amino acid	Wavelength maxima (nm)	$\epsilon \times 10^{-3}$ (l/mol cm)
Cysteine	250	0.3
Histidine	211	5.9
Phenylalanine	188	60.0
	206	9.3
	257	0.2
Tryptophan	219	47.0
	279	5.6
Tyrosine	193	48.0
	222	8.0
	275	1.4

^aValues are for aqueous solutions at pH 7.1 (Freifelder, 1982; Fasman, 1989).

Table 10.1A.2 Molar Absorptivity (ϵ) of Aromatic Amino Acids at 280 nm^a

Amino acid	$\epsilon \times 10^{-3}$ (l/mol cm)
Phenylalanine	0.0007
Tryptophan	5.559
Tyrosine	1.197

^aValues are for aqueous solutions at pH 7.1 (Fasman, 1989).

somewhat at that wavelength. The ϵ_{280} for tryptophan is nearly 5-fold greater than that for tyrosine (Table 10.1A.2). Several amino acids other than those in Table 10.1A.1 absorb light below 205 nm (Fasman, 1989), but either the molar absorptivities are too low to be significant or the wavelengths are too short for practical absorbance measurements.

The aromatic amino acids also exhibit fluorescence emissions when excited by light in the UV range. Table 10.1A.3 gives the excitation wavelength, fluorescence emission wavelength, and quantum yield (Q) for tryptophan, tyrosine, and phenylalanine. The quantum yield is the ratio of photons emitted to photons absorbed. Typically, phenylalanine fluorescence is not detected in the presence of tyrosine and tryptophan due to low Q . Furthermore, tyrosine fluorescence is nearly completely quenched if the tyrosine residue is ionized or near an amino group, a carboxyl group, or a tryptophan residue (Teale, 1960; Freifelder, 1982). Therefore, tryptophan fluorescence is what is customarily measured.

Measurement of intrinsic fluorescence by aromatic amino acids is primarily used to ob-

tain qualitative information (Freifelder, 1982). However, with a protein standard whose aromatic amino acid content is similar to that of the sample, intrinsic fluorescence can be used for quantitation (Hawkins and Honigs, 1987). An additional consideration is that the tertiary structure of a protein will influence the fluorescence, e.g., adjacent protonated acidic groups in a protein molecule will quench tryptophan fluorescence (Freifelder, 1982).

The most frequently employed colorimetric methods for determining protein concentration are those of Bradford (1976) and Lowry et al. (1951). The Bradford method, which is faster than the Lowry method, is the method of choice for determining protein concentration. It is possible to miniaturize the Bradford assay by using a 96-well microtiter plate assay as described by Brogdon and Dickinson (1983).

The Lowry method (as modified by Peterson, 1977) is dependent on the unknown and the standard protein having a similar content of tyrosine per microgram protein. If the protein has fewer (or more) tyrosines/ μ g than BSA, then the protein concentration determined will be too low (or too high). This method is not

Table 10.1A.3 Fluorescence Properties of Aromatic Amino Acids^a

Amino acid	Excitation wavelength	Emission wavelength	Quantum yield
Phenylalanine	260 nm	283 nm	0.04
Tryptophan	285 nm	360 nm	0.20
Tyrosine	275 nm	310 nm	0.21

^aValues are for aqueous solutions at pH 7 and 25°C (Hawkins and Honigs, 1987; Fasman, 1989).

appropriate for proteins without tyrosine residues, since the assay relies on the reaction of these residues with the reagent.

Critical Parameters and Troubleshooting

A 1-cm-path-length quartz cuvette is most often used for spectrophotometric detection. However, quartz cuvettes with shorter path lengths (0.01 to 0.5 cm) are available (e.g., from Hellma Cells or Beckman); these allow higher concentrations of protein solutions to be measured. The equations relating absorbance and protein concentration (see Basic Protocol 1 and Alternate Protocol 1) assume the cuvette has a path length of 1 cm; when cuvettes of shorter path length are used, the correct value for *b* must be substituted in the equation.

Glass cuvettes can be used for colorimetric detection. Quartz cuvettes are not recommended for the Bradford assay because the dye adheres more readily to quartz than to glass.

Spectrophotometric detection

The solvent pH and polarity will affect the absorbance and fluorescence properties of a protein. A notable example of pH effects on absorbance is seen with tyrosine residues, where a change in pH from neutral to alkaline results in a shift of the absorbance maximum to a longer wavelength and an increase in absorptivity due to dissociation of the tyrosine phenolic hydroxyl group (Freifelder, 1982; Fasman, 1989). An example of solvent polarity effects on fluorescence is that observed with tryptophan, where a decrease in solvent polarity results in a shift in fluorescence emission to shorter wavelengths and an increase in intensity (Freifelder, 1982). Because of these effects, the following precautions should be taken for accurate results: (1) when calibration curves are used for quantitation by absorbance or fluorescence, standards must be in the same solvent as the samples; and (2) when a published absorptivity at a given wavelength is used for quanti-

tation, the solvent composition of the sample must be the same as that used in obtaining the published data.

Many buffers and other reagents can interfere with *A*₂₈₀ and *A*₂₀₅ spectrophotometric measurements. Stoscheck (1990) lists the concentration limits for many such reagents used in these spectrophotometric methods. The more commonly used reagents that absorb at 280 and 205 nm are listed in Table 10.1A.4. In addition, reagents that contain carbon-carbon or carbon-oxygen double bonds can interfere with the *A*₂₀₅ method.

Because stray light can affect the linearity of absorbance versus concentration, absorbance values >2.0 should not be used for sample proteins measured by the *A*₂₈₀ or *A*₂₀₅ method. Samples with absorbance >2.0 should be diluted further in the appropriate buffer to obtain absorbances <2.0.

Nucleic acids have substantial absorbance at 280 nm and can interfere with *A*₂₈₀ quantitation of protein in crude samples. To resolve the protein concentration in such samples, measure the absorbance at 260 nm and 280 nm and calculate the protein concentration as follows (Warburg and Christian, 1942; Layne, 1957): protein concentration (mg/ml) = $1.55 \times A_{280} - 0.76 \times A_{260}$.

This estimation of protein concentration is valid up to 20% (w/v) nucleic acid or an *A*₂₈₀/*A*₂₆₀ ratio <0.6.

Colorimetric detection

Many substances will interfere with the Bradford protein assay, including glycerol, detergents, 2-mercaptoethanol, acetic acid, ammonium sulfate, Tris, and certain alkaline buffers, but the appropriate controls can be used to correct this interference.

Many substances will interfere with the Lowry protein assay (Peterson, 1979). If detergents, denaturants, organic buffers, and/or thiols are present in the unknown protein solution, it is important to assess their effects on

Table 10.1A.4 Concentration Limits of Interfering Reagents for A_{205} and A_{280} Protein Assays^a

Reagents ^b	A_{205}	A_{280}
Ammonium sulfate	9% (w/v)	>50% (w/v)
Brij 35	1% (v/v)	1% (v/v)
DTT	0.1 mM	3 mM
EDTA	0.2 mM	30 mM
Glycerol	5% (v/v)	40% (v/v)
KCl	50 mM	100 mM
2-ME	<10 mM	10 mM
NaCl	0.6 M	>1 M
NaOH	25 mM	>1 M
Phosphate buffer	50 mM	1 M
SDS	0.10% (w/v)	0.10% (w/v)
Sucrose	0.5 M	2 M
Tris buffer	40 mM	0.5 M
Triton X-100	<0.01% (v/v)	0.02% (v/v)
TCA	<1% (w/v)	10% (w/v)
Urea	<0.1 M	>1 M

^aValues from Stoscheck (1990).

^bAbbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

absorbance by creating standard curves based on data taken in both the presence and the absence of these compounds. Precipitation of a protein with deoxycholate/trichloroacetic acid will eliminate many of these interfering substances while allowing a quantitative recovery of cytosolic and membranous proteins (Peterson, 1979). Although the standard curve for BSA is nonlinear, the simplicity, sensitivity, and reproducibility of the method make it worthwhile. The A_{750} will decrease at a rate of 1% to 2% per hour at room temperature.

If the unknown protein solution contains detergents such as 1% Triton X-100, 1% Tween 20, 1% Nonidet P-40, or 0.75% cetyltrimethylammonium bromide, the addition of ~4% SDS will prevent precipitation of the Folin reagent (Cadman et al., 1979).

Anticipated Results

Depending on the protein, the concentration range for the A_{280} method is 20 to 3000 $\mu\text{g/ml}$, for the A_{205} method is 1 to 100 $\mu\text{g/ml}$, and for the fluorescence emission method is 5 to 50 $\mu\text{g/ml}$.

Published absorptivities of proteins at 280 nm are usually given as the absorbance for a 1% (w/v) protein solution per cm, $A^{1\%}$, or as the molar absorptivity, ϵ , which has units of l/mol cm . To convert these published coefficients to units of mg/ml , use one of the following equations:

$$\text{concentration (mg/ml)} = \frac{A_{280} \times 10}{A^{1\%} \times b} \text{ or}$$

$$\text{concentration (mg/ml)} = \frac{A_{280} \times \text{molecular weight}}{\epsilon_{280} \times b}$$

Depending on the reagent volumes used, it is possible to quantitate protein in the range of 1 to 100 μg using the Bradford or Lowry assay.

Time Considerations

When the absorptivity for a protein is known, the A_{280} and A_{205} measurements require <30 min depending on the number of samples. When standards are used for quantitation with these assays or for intrinsic fluorescence quantitation, 1 hr is required.

The Bradford method requires ~30 min. The Lowry method requires 2 hr to generate a standard curve and to run several unknown protein samples.

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Detailed discussion of intrinsic fluorescence of proteins and what factors affect fluorescence emission by the aromatic amino acids (see pp. 618-663).

Darbre, A. 1986. Analytical methods. In *Practical Protein Chemistry: A Handbook* (A. Darbre, ed.) pp. 227-335. John Wiley & Sons, New York.

Describes colorimetric methods for protein determination including the Lowry method and several modifications, the biuret method and several modifications, and the Bradford method (see pp. 284-295).

Fasman, G.D. 1989. See above.

Contains tables with absorptivities for UV spectrophotometric detection and tables with data on excitation and emission wavelengths for fluorescence detection of many proteins. Also includes a table with molecular weights for many characterized proteins.

Stoscheck, C.M. 1990. See above.

Contains list of substances that can interfere with 205- and 280-nm spectrophotometric and colorimetric measurements of proteins and of concentration limits for these substances.

Contributed by Michael H. Simonian
(spectrophotometric methods)
Beckman Coulter, Inc.
Fullerton, California

John A. Smith (colorimetric methods)
University of Alabama at Birmingham
Birmingham, Alabama

Glucose ENFET doped with MnO₂ powder

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Abstract

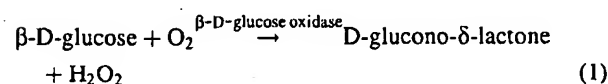
In this study, a glucose-sensitive enzyme field-effect transistor (ENFET) coimmobilized with glucose oxidase (GOD) and manganese dioxide (MnO₂) have been investigated. The biomembrane of the ENFET was immobilized on the amorphous tin oxide/indium tin oxide glass structure extended sensitive gate, which used as a disposable transducer of a glucose biosensor. MnO₂ was used as a catalyst which can catalyze the hydrogen peroxide and produced H₂O and O₂. Coimmobilization of glucose oxidase and manganese dioxide was found to be useful for extending the dynamic measured range of glucose concentration to 360 mg/dl (eq. 20 mM). The result shows that the dynamic range of the output signal is strongly dependent on pH value of measuring environments, and the measurement in the alkali buffer solutions shows a higher response and wider dynamic range. Additionally, the different immobilized layers of MnO₂ have been studied. The MnO₂, which be immobilized in outer cross-linking layer of bovine serum albumin, shows better results than immobilized in GOD layer or glutaraldehyde covalent layer. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Extended gate field effect transistor (EGFET); Enzyme field effect transistor (ENFET); Amorphous tin oxide; Glucose oxidase (GOD); Manganese dioxide (MnO₂)

1. Introduction

Since the first reported enzyme biosensor (ENFET) based on ion-sensitive field effect transistors (ISFETs) [1], substantial research efforts were undertaken to improve the performance characteristics of the ENFETs developed. Until now, there are almost two dozen papers dealing with glucose ENFETs which suffer from many problems [2].

Normally glucose oxidase hydrolyzes glucose according to the following reactions:



ISFET sensors measure the glucose concentration by detecting the pH variation due to the hydrogen ions that are

generated by the dissociation of gluconic acid. However, because of the low dissociation constant ($\text{pK}_a \approx 3.8$) [3], ISFET glucose sensors show low sensitivities. Generally, the sensitivities at the physiological pH value are limited to only some millivolts per decade [4]. Hence the ISFET drift, which is an inherent characteristic of ISFETs, becomes a important topic. The glucose concentration in human blood is normally about 5 mM, reaching 20 mM and more for diabetics. However, the concentration of oxygen, does not exceed 0.5 mM. Because of the unfavourable concentration ratio of glucose and oxygen in real blood, the dynamic range of the biosensor is usually limited by oxygen and does not exceed several mM. Since, the oxygen in the sensor membrane is consumed by the enzyme reaction, the oxygen concentration is needed high enough for a better linearity between output voltage and the glucose concentration. Moreover, the hydrogen peroxide, one of the by-products of the glucose oxidation, acts an inhibitor of glucose oxidase which causes the lower sensitivity and bad repeatability in the steady measurement system of glucose ENFET.

Sudoh et al. employed pre-electrolysis method to enrich the oxygen of the glucose solution, which the oxygen is generated by electrolysis of the solution before monitoring

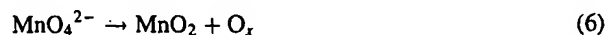
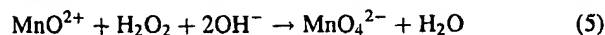
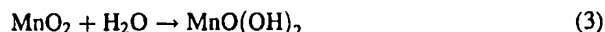
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[5]. The output signal was linearly proportional to the glucose concentration up to 500 mg/dl by the supplement of oxygen generated by electrolysis of the solution. Seo et al. and Lee et al. employed a Pt electrode actuator on the ISFET sensitive gate to electrolyze the hydrogen peroxide [2,6]. The sensor with the Pt electrode actuator exhibits a wide dynamic range that from 1 to 10 mM. Saito et al. used an external BSA membrane, which is highly cross-linked by glutaraldehyde, to restrict glucose diffusion to expand the measuring range and make a stable response in a low buffer capacity solution [7]. The sensor outputs shows a good linear relationship with up to 300 mg/dl glucose concentration. Shul'ga et al. added the potassium ferricyanide, which used as an oxidizing substrate in the biocatalytic oxidation of glucose, into the phosphate and TRIS buffer to perform the glucose ENFET measurement [8]. Depending on the concentration of ferricyanide the glucose ENFET shows a 10–100 times increase of the biosensor response and a substantial extension of its dynamic range.

In this paper a glucose sensor based on H^+ -ion-sensitive field effect transistor (ISFET) has been realized in combination with a MnO_2 -doped glucose oxidase membrane. Zheng and Guo brought forward the following procedures and reactions of H_2O_2 catalyzed by MnO_2 [9]:



Where MnO_2 was used as a catalyst which can catalyze the hydrogen peroxide and produce H_2O and O_2 . In addition to the reduction of H_2O_2 concentration in the biolayer, the product, oxygen, can be recycled for glucose oxidation reaction. The MnO_2 doping position and the pH value of working have been investigated.

2. Experimental

2.1. Chemicals and materials

The β -D-glucose oxidase (GOD) EC 1.1.3.4 from *Aspergillus niger*, bovine serum albumin from Serva and γ -aminopropyl triethoxysilane (3-APTS, 99%) were purchased from Sigma. Glutaraldehyde (GA, 25% aqueous solution) was purchased from Acros Organics. Manganese dioxide powder (99.9%) was obtained from Tekstart (Hsinchu, Taiwan). All other reagents were in reagent grade and were used without further purification. Distilled water was used for all the electrolytes and the buffer solutions. Tin oxide thin films were formed by the RF sputtering system (tin oxide target, 99.9%) at a substrate temperature of 150°C. The ITO glasses (50–100 Ω /sq; ITO coating thickness, 230 Å) were supplied by the Wintek Corporation.

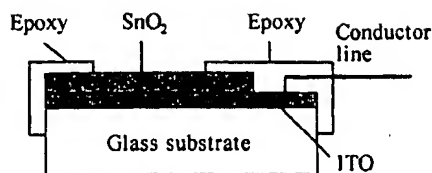


Fig. 1. Cross-section of SnO_2 /ITO glass sensing structure.

2.2. Sensor Fabrication

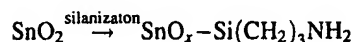
2.2.1. Solid-state part

In this study, the glucose ENFET is based on a separative extended gate ISFET (EGFET) structure. The sensitive part of the separative EGFET is shown as Fig. 1. The SnO_2 thin film was deposited by using sputtering method with a thickness of 2000 Å. Before the glass was deposited SnO_2 , it was washed in methyl alcohol and DI water for 20 and 10 min, respectively. The SnO_2 /ITO glass EGFET shows a linear pH response about 57 mV/pH between pH 2.4 and pH 11.2 [10].

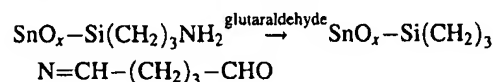
2.2.2. Enzyme immobilization

The procedure for preparation of separative structure of ENFET is as follows.

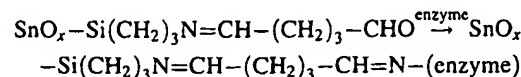
1. Cleaning: The separative structure of EGFET was cleaned by distilled water.
2. Silanization: There is no amino group on our sensitive film, so we use 3-APTS to modify tin oxide (SnO_2) substrate. The procedure is represented as follow [11,12]:



3. Activation by glutaraldehyde: Glutaraldehyde (1%) is also used extensively to immobilize enzyme molecules onto a carrier substance bearing amino group. The procedure is represented as follow:



4. Coupling of the enzyme and cross-linking: The GOD (40 mg) was dissolved in 1 ml of a 0.1 M K-P buffer solution (pH 7.0). A 1.5 μ l part of the solution was cast onto the gate region and then addition of 1 μ l of the glutaraldehyde was followed to chemically cross-link the membrane. The procedure is represented as follow:



5. The outer BSA membrane doping with MnO_2 : An amount of 10 mg MnO_2 was dissolved in 300 mg/dl BSA and 6% glutaraldehyde (1:1) solution. A 1 μ l part of the solution was cast onto the enzyme membrane.

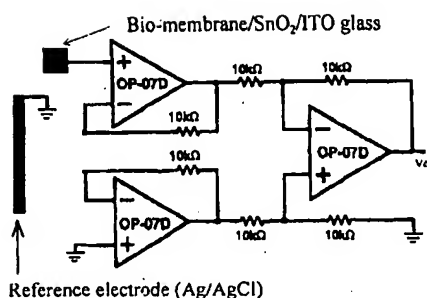


Fig. 2. Separative extended ENFET measurement circuit with instrumentation amplifier LT1167.

In the study of the effect of MnO_2 doping position, the outer BSA membrane was not immobilized, and the MnO_2 was doped in activation or GOD with 10 mg/ml MnO_2 .

2.3. Measurement

A readout circuit based on an instrumentation amplifier LT1167 is shown in Fig. 2. The measurement configuration consists of a separative extended gate with biomembrane and the Ag/AgCl reference electrode. The instrumentation amplifier, LT1167, was a transducer and the small output voltage will depend on the pH value. HP3478A and HP VEE program were designed and used as an Y–T recorder to record the voltage variation with time. All measuring temperature of our experiments are in 25°C, 5 mM phosphate–KOH buffer.

3. Results and discussion

3.1. Glucose ENFET response

In this study, a separative sensitive gate of biomembrane/ SnO_2 /ITO glass structure was used as a disposable biochemical transducer. This structure has advantages of light insensitivity, easier fabrication processes than traditional ISFET and lower cost than SOS structure ISFETs or silicon based EGFET [10]. Fig. 3 shows the pH response of separative sensitive structure with biomembrane of sensitivity 58.3 mV/pH between pH 2 and 10.

Figs. 4 and 5 show typical time response curve for the glucose ENFET without and with the outer BSA membrane. The glucose ENFET was immersed in blank buffer solution for 1 min and then immersed in glucose solution. As the ENFET are measured in blank buffer, it shows a drift that not exceeds to 1 mV for 1 min. The glucose ENFETs without and with BSA membrane show response time of 5 and 12 min, respectively. The glucose ENFET, which has outer MnO_2 -doped BSA membrane, shows a good linearity up to 360 mg/dl, because the O_2 concentration in biomembrane was greatly rose by MnO_2 catalyzing H_2O_2 . The relationship between the sensor output and glucose concentration is shown in Fig. 6.

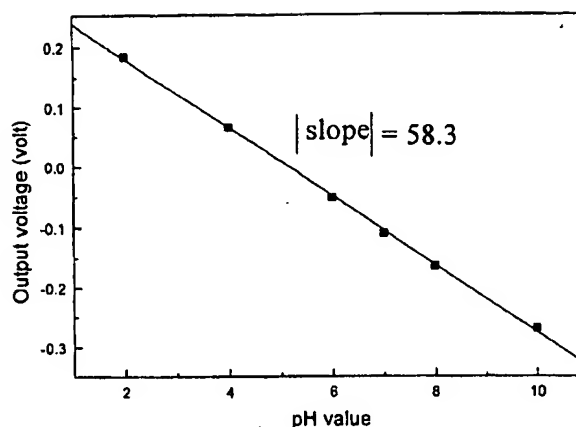


Fig. 3. Output voltage vs. pH value for the biomembrane/ SnO_2 /ITO glass sensing gate connected with instrumentation amplifier LT1167.

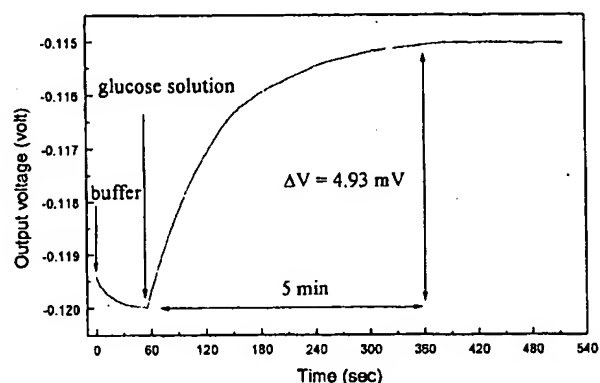


Fig. 4. Response of the separative structure of the ENFET without outer BSA membrane to detect 40 mg/dl glucose in pH 7.2 buffer solution. MnO_2 was immobilized in GOD layer.

Saito et al. employed the glucose ENFET with external BSA membrane, which is very similar our device, shows a good linear relationship with up to 300 mg/dl glucose concentration [7] which the experiments are performed in a

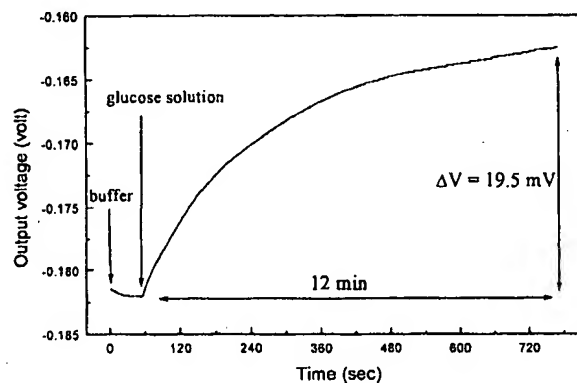


Fig. 5. Response of the separative structure of the ENFET with outer BSA membrane to detect 45 mg/dl glucose in pH 8.5 buffer solution. MnO_2 was immobilized in BSA layer.

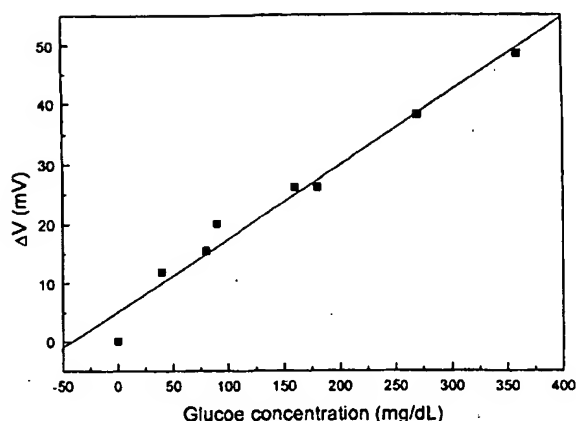


Fig. 6. Calibration curve for the glucose ENFET with outer MnO_2 -doped BSA membrane. Sensors were measured in pH 8.1, 5 mM buffer solution.

stirring status. But, all the measurements in this paper are performed in a steady status. Fig. 7. shows the sensor response for sensors with non-doped MnO_2 outer BSA layer and MnO_2 -doped BSA layer. The results show that sensors with MnO_2 -doped BSA show wider dynamic range than sensors with non-doped MnO_2 outer BSA layer. The sensors of non- MnO_2 -doped show a high response in lower glucose concentration, but very low response in high glucose concentration.

3.2. Effect of pH on the ENFET response

According to the report of Zheng and Guo, in the experiment of potentiometric determination of hydrogen peroxide at MnO_2 -doped carbon paste electrode, while the pH changed in the range 7.0–8.0, the potential response increased with increasing pH [9]. The results may be related to the enhancing of oxidizing ability of H_2O_2 when pH changed in this range. For pH values in the range of 8.0–9.0, the

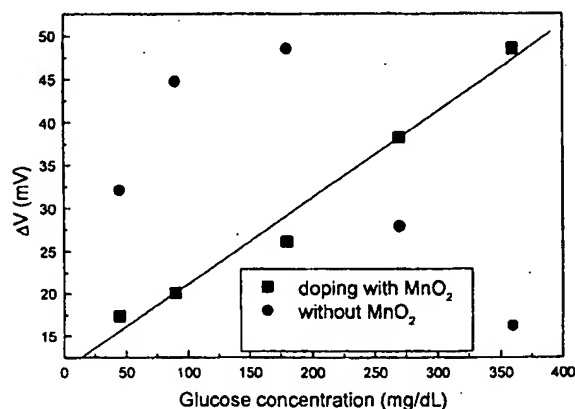


Fig. 7. Calibration curve for the glucose ENFET sensors with (●) non-doped MnO_2 outer BSA layer and (■) MnO_2 -doped BSA layer. Sensors were measured in pH 8.1, 5 mM buffer solution.

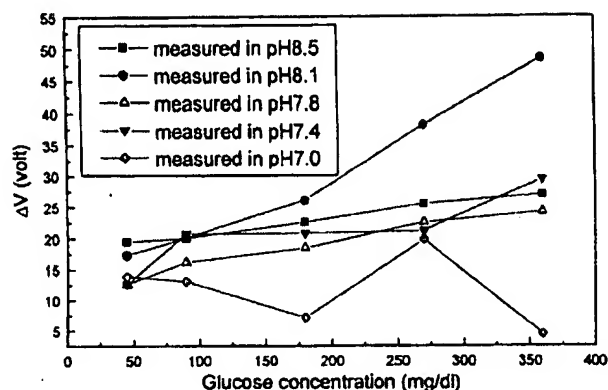


Fig. 8. Effect of pH on the glucose ENFET with outer MnO_2 -doped BSA membrane. The curves correspond to different pH values of measured environments: (■) pH 8.5; (●) pH 8.1; (▲) pH 7.8; (▼) pH 7.4; (◆) pH 7.0.

response was almost constant. In our research, the effect of pH on the glucose ENFET response shows the best results of sensitivity and linearity that are shown in Fig. 8. As the GOD catalyzes glucose, the actual pH value of the ENFET biomembrane is lower than the pH value of buffer. The output signal of the measurement in pH 8.5 is lower than that of in pH 8.1 which is caused by that the activity of the GOD is bad in alkali [13,14]. In addition, the results measured in lower pH environments show a bad linearity which is caused by that the MnO_2 shows lower catalysis ability in acid.

3.3. Effect of MnO_2 doping position

As mention before, while the pH changed in the range 7.0–8.0, the catalysis ability of MnO_2 increased with increasing pH value. However, the actual pH value is different in individual biomembrane layer as the ENFET dips into glucose solution. The GOD layer shows the lowest pH that caused by the glucose catalyzed and producing H^+ -ion. The H^+ will diffuse into the activation layer and outer layer, which close to the SnO_2 sensitive film and pH-buffer

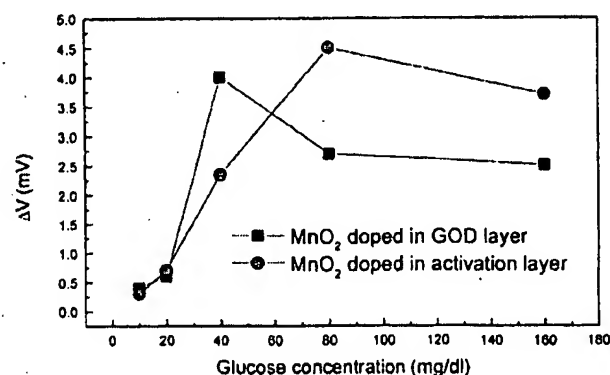


Fig. 9. Glucose concentration response of the ENFET that MnO_2 -doped in the GOD layer and in the activation layer, respectively.

solution, respectively. By the effect of the carrier-mediated transport of protons, the outer membrane will show the highest pH value, which is better suitable condition for the reaction between MnO_2 and H_2O_2 . As the results shown above, the glucose ENFET that biomembrane with outer MnO_2 -doped BSA membrane has a linear dynamic range to 360 mg/dl. Fig. 9. shows the glucose response of MnO_2 -doped in the activation layer and the GOD layer. The responses are limited in the high glucose concentration, which caused by that the reaction between MnO_2 and H_2O_2 was blocked in acid environments, especially the response of the device was MnO_2 -doped in enzyme layer.

4. Conclusions

A glucose ENFET based on a new principle, in which the biomembrane was doped with MnO_2 powder. MnO_2 was used to perform the catalysis of hydrogen peroxide (one of the by-products of glucose oxidation), was proposed and its characteristics were investigated. The sensor shows a wide dynamic range to the glucose concentration of 360 mg/dl. Both the pH value of buffer solution and MnO_2 -doped position affect the response of the glucose ENFET. For the MnO_2 -doped effect, both the responses of MnO_2 -doped in the activation layer and the GOD layer are limited in the high glucose concentration, which is caused by that the reaction between MnO_2 and H_2O_2 blocked in acid environments, especially the response of the device that MnO_2 -doped in the enzyme layer. For the effect of pH value of buffer solution, the glucose ENFET, which MnO_2 -doped in the outer BSA layer, measured in pH 8.1 has the largest response and the widest dynamic range in our experiments. In addition, the sensors with MnO_2 -doped BSA show wider dynamic range than sensors with non-doped MnO_2 outer BSA layer.

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